University of Alberta

GROWTH HORMONE IN THE DEVELOPING VISUAL SYSTEM: DISTRIBUTION AND ROLES

by

Marie-Laure Baudet ©

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To Gilles, Annick, Nicolas & David

Abstract

Postnatally, growth hormone (GH) is primarily produced in the anterior pituitary and promotes growth and metabolism. The prenatal roles for GH have been hitherto overlooked, mostly because pituitary GH is not present during early gestation. GH has, however, been recently detected within extrapituitary tissues of early chick embryos including the neural retina. The possibility that the visual system is a local site of GH production and action during early development was thus investigated.

GH mRNA was mostly found in the retinal ganglion cell (RGC) layer of the E7 retina. Similarly, GH-immunoreactivity was detected within the cytoplasm of RGCs and in their projecting axons. GH was also abundant in the tectobulbar tract of the optic tectum, the target tissue of RGC axons. GH receptor (GHR) expression mirrored that of GH within RGCs and their axons, and in the optic tectum, suggesting that RGCs axons and the tectobulbar tract are autocrine sites of GH action. Exogenous GH was found to promote axon growth of immunopanned RGC culture at 10^{-6}M and 10^{-9}M.

A novel variant of GH, small chicken GH (scGH), was also shown to be present in the visual system, but its distribution differed from that of GH. Using *in vitro* transcription and western blotting, the translation of the scGH transcript was demonstrated. Immunoneutralization of scGH increased the number of apoptotic cells, suggesting neuroprotective role for scGH. scGH might mediate its biological activity via a novel class of receptors, as structural modeling of scGH suggests it is unlikely to bind to GHR.

GH mRNA and protein were also found in the RGC layer of newborn mouse. Using 2D gel analysis and mass spectrometry, BASP-1 was found to be downregulated

whereas PKC inhibitor-1, Cyclophilin-A, and SLM-2 were upregulated in the neural retina of GHR knockout compared to wild-type mice. These proteins have been implicated in axon growth, synaptogenesis, vascularization, and cell proliferation suggesting putative roles for GH in the regulation of these processes in the developing neural retina of mice.

Overall, this research establishes that the neural retina of embryonic chicks and newborn mice is a local site of GH production and action.

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List of Abbreviations

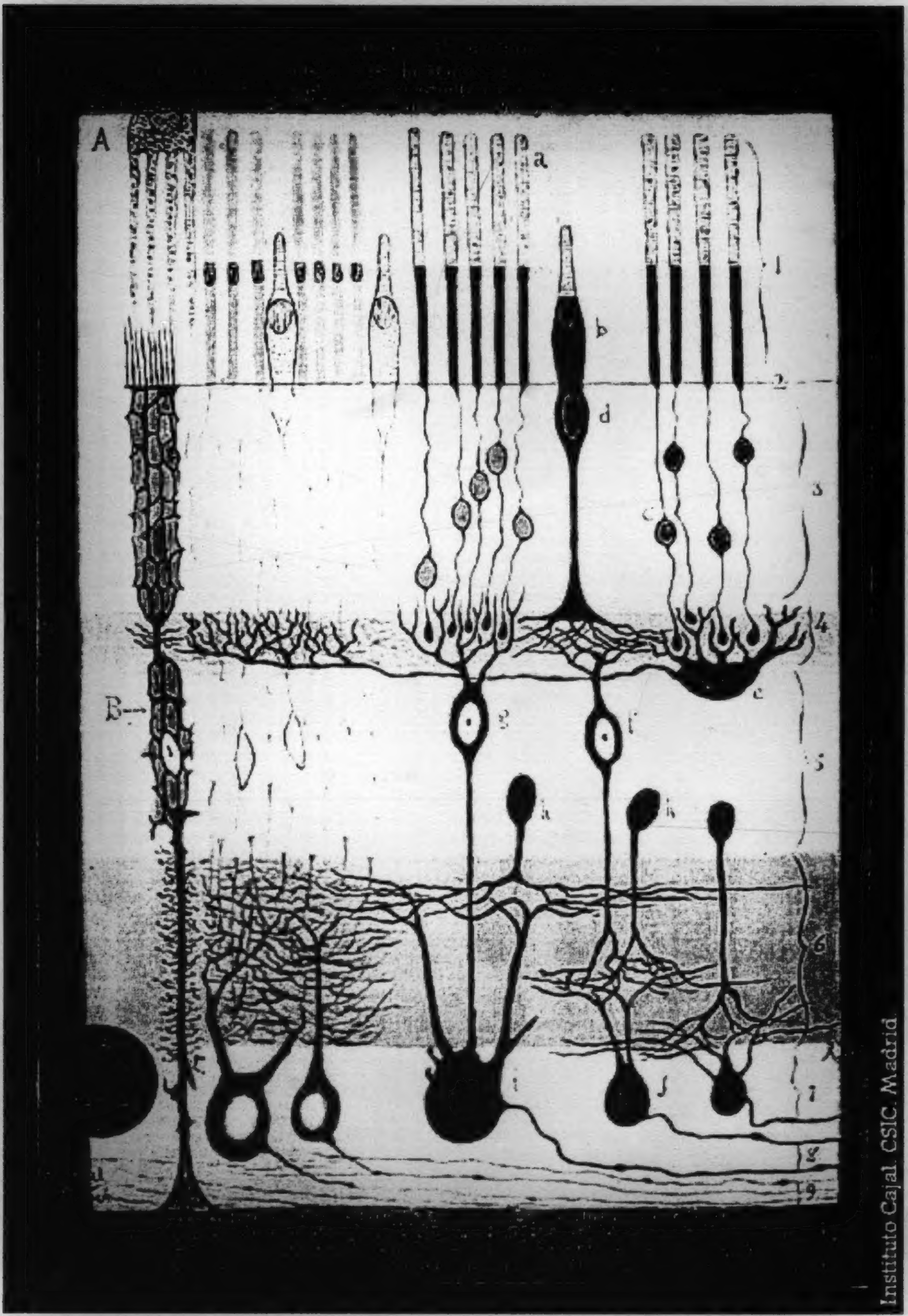
ABC	Avidin:Biotinylated enzyme Complex
ALS	Acid Labile-Subunit
AIF	Apoptosis Inducting Factor
APP	Amyloid Precursor Protein
BASP-1	Brain Abundant, membrane attached Signal Protein-1
BAB	Blood Aqueous Barrier
BBB	Blood Brain Barrier
BBG	Bovine Brain Gangliosides
BDNF	Brain Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
bGH	bovine Growth Hormone
bHLH	basic Helix Loop Helix
BMP	Bone Morphogenetic protein
BOB	Blood Ocular Barrier
bp	base pair
BRB	Blood retinal Barrier
BrdU	Bromodeoxyuridine
BMP	Bone Morphogenetic Protein
cGH	Chicken GH
CHO	Chinese Hamster Ovary
CL	Choroid Layer
CNTF	Ciliary Neurotrophic Factor
CNS	Cental Nervous System
Da	**Dalton**
DAB	Diaminobenzidine Tetrahydrochloride
DE	2D Electrophoresis
E	Embryonic Day
ECD	Extracellular Domain
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELM	External Limiting Membrane
ER	Endoplasmic Reticulum
FGF	Fibroblast Growth Factor
FIF	Fibroblast Growth Factor Interacting Factor
GCL	Ganglion Cell Layer
GDNF	Glial Cell Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GH	Growth Hormone
GHBP	Growth Hormone Binding Protein
GHD	Growth Hormone Deficiency
GHR	Growth Hormone Receptor
GHRH	Growth Hormone Releasing Hormone
HGF	Hepatocyte Growth Factor

hGH	Human Growth Hormone
IEF	Isoelectric Focusing
IGF	Insulin-like growth factor
IGF-BP	Insulin-like Growth Factor-Binding Protein
IL	Interleukin
INL	Inner Nuclear Layer
iNOS	inducible Nitric Oxide Synthase
IPG	Immobilized pH Gradient
IPL	Inner Plexiform Layer
IZ	Intermediate Zone
JAK	JAnus Kinase
K / kDa	Kilo dalton
LB	Luria Bertani
LIF	Leukaemia Inhibitory Factor
lON	left Optic Nerve
LPOS	Layer of Photoreceptor Outer Segments
mGH	mouse GH
MS	Mass Spectrometry
MZ	Marginal Zone
NB	Neuroblastic Layer
nc	Notochord
N-CAM	Neural Cell Adhesion Molecule
NGF	Nerve Growth Facror
NGS	Normal Goat Serum
NLS	Nuclear Localization Signal
NMS	Normal Mouse Serum
NOS	Nitric Oxide Synthetase
NR	Neural Retina
NRS	Normal Rabbit Serum
NT	Neurotrophin
OC	Optic Chiasm
OFL	Optic Fiber Layer
ON	Optic nerve
OPL	Outer plexiform layer
OT	Optic tectum
OTr	Optic Tract
P	Postnatal day
p	pia / pial surface
PARP	Poly (ADP-ribose) Polymerase
PDB	Protein Data Base
Pit-1	PItuitary specific Transcription Factor-1
PNS	Peripheral Nervous system
PRL	Prolactin
RGC	Retinal Ganglion Cell
rGH	rat Growth Hormone
rON	right Optic Nerve

RPE	Retinal Pigmented Epithelium
RPTP	Protein Tyrosine Phosphatase
SAC	*Stratum Album Central*
scGH	small chicken GH
SFP	*Stratum Fibrosum*
SGC	*Stratum Griseum Centrale*
SGFS	*Stratum Griseum et Fibrosum Superficiale*
SGP	*Stratum Griseum Periventiculare*
Shh	Sonic hedgehog
Spi	Serine Protease Inhibitor
SO	*Stratum Opticum*
SOCS	Suppressors of Cytokine Signaling
SRIF	Somatrotopin-Release Inhibitory Factor
STAT	Signal Transducers and Activators of Transcription
TGF	Transforming Growth Factor
TK pA	Thymidine Kinase polyAdenosine
TP	Tectal Plate
UTR	Untranslated region
VEGF	Vascular Epidelium Growth Factor
v	vitreous
VZ	Ventricular Zone

Chapter 1

Literature review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Instituto Cajal. CSIC. Madrid.

"Structure of the Mammalian Retina" c.1900 By Santiago Ramón y Cajal

Preamble

Visual stimuli are sensed by photoreceptors in the neural retina and transduced into electrical information, which is then conveyed through retinal ganglion cell (RGC) projections via the optic nerve to the visual processing centers in the brain. While the visual system has been widely studied, the possible involvement of growth hormone (GH) in its development has rarely been considered. Until recently, GH was regarded as an endocrine released from the pituitary gland into the systemic circulation that regulates growth and metabolism in the body. However, with the discovery that GH expression is not restricted to the pituitary gland and is almost ubiquitous during development, it is now considered to be a tissue growth factor with local autocrine or paracrine actions. The presence and action of GH in the developing visual system is the focus of my thesis research and the background literature on which it is based is the subject of the following review.

1.1 The visual system

In this section, the literature pertaining to the development of neural tissues of the visual system (the neural retina and the avian optic tectum) will be discussed.

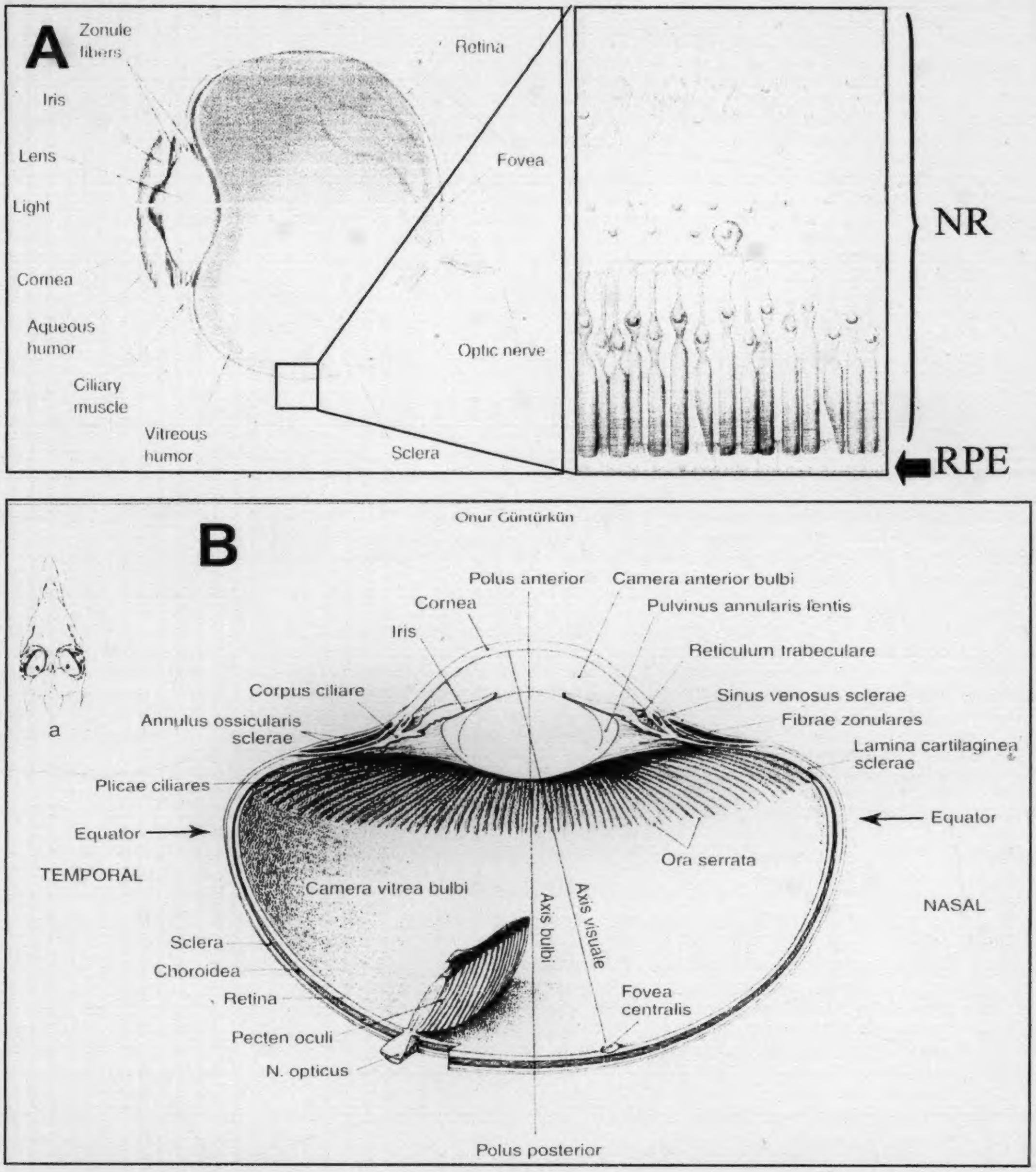
1.1.1 Anatomy of the visual system

1.1.1.1 Anatomy of the neural retina

As reviewed by Bear et al. (2001) and described in detail by Ramón y Cajal (1893), the vertebrate retina, located at the back of the eye, is divided into the neural retina and the retinal pigmented epithelium (Fig. 1.1). The neural retina is further divided into nuclear and plexiform layers (Fig. 1.2) and has a relatively simple laminated structure. Six types of neurons only compose the neural retina: rod and cone photoreceptors are sensory neurons and transduce the light into membrane potentials, bipolar, horizontal and amacrine cells are interneurons and retinal ganglion cells (RGCs) are the projecting neurons of the retina that convey electrical activity to the brain. RGC axons constitute the optic fiber layer (OFL) within the retina (Bear et al., 2001), which is the innermost layer of the neural retina, adjacent to the RGCs. The laminar structure of the neural retina is formed progressively during development (Fig. 1.3).

In addition, the neural retina of vertebrates contains three types of glial cells. Müller cells are the main glial cells of the retina, they are derived from retinal progenitors and they span the width of the neural retina (Bringmann et al., 2006). In adults, Müller cells maintain retinal homeostasis, process neurotransmitters and have metabolic and structural roles (Barnstable, 2004; Bringmann et al., 2006). Oligodendrocytes migrate into the retina during development and myelinate RGC axons in the optic fiber layer of the retina in chicken but not in most mammals, and in the optic nerve (Mey and Thanos, 2000; Won et al., 2000). Finally, microglia cells also have an extraretinal origin and act as retinal immune cells (Chen et al., 2002). Astrocytes, a fourth type of glial cells, are also present in species with vascularized retinas such as rodents, and often ensheath blood vessels (Won et al., 2000; Bringmann et al., 2006).

In adult fish and amphibians, retinal progenitors are located within a circumferential germinal zone (CGZ) at the ciliary margin, between the retina and the iris epithelium, and provide neural cells for continued growth and regeneration (Reh and Levine, 1998; Otteson and Hitchcock, 2003). In addition in fish, rare stem cells are also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Iris
Lens
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Cornea
Aqueous humor
Ciliary muscle
Vitreous humor
Retina
Fovea
Optic nerve
Sclera
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RPE
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Onur Güntürkün
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Polus anterior
Camera anterior bulbi
Cornea
Pulvinus annularis lentis
Iris
Reticulum trabeculare
Corpus ciliare
Sinus venosus sclerae
Annulus ossicularis sclerae
Fibrae zonulares
Lamina cartilaginea sclerae
Plicae ciliares
Equator
Equator
Ora serrata
TEMPORAL
Camera vitrea bulbi
NASAL
Axis bulbi
Axis visuale
Sclera
Choroidea
Retina
Fovea centralis
Pecten oculi
N. opticus
Polus posterior

Fig. 1.1. Eye anatomy (**A**) The left panel represents a cross section of a human eye and the right panel represents a higher magnification of the retina. The retina is composed of two layers, the neural retina (NR) and the retinal pigmented epithelium (RPE). Modified and reprinted from Neuroscience, Exploring the brain, second edition, M Bear, B Connors & M Paradiso, "The Eye", p286 & p292, Copyright (2001), with permission from Lippincott Williams & Wilkins). (**B**) The chicken eye is shown for comparison. The chicken eye differs from the mammalian eye in that its vascularization is confined to the pecten oculi, a structure that protrudes into the vitreous. Reprinted from Sturkie's Avian Physiology, Second Edition, O Güntürkün, Sensory Physiology: Vision, page 2, Copyright (2000), with Permission from Elsevier.

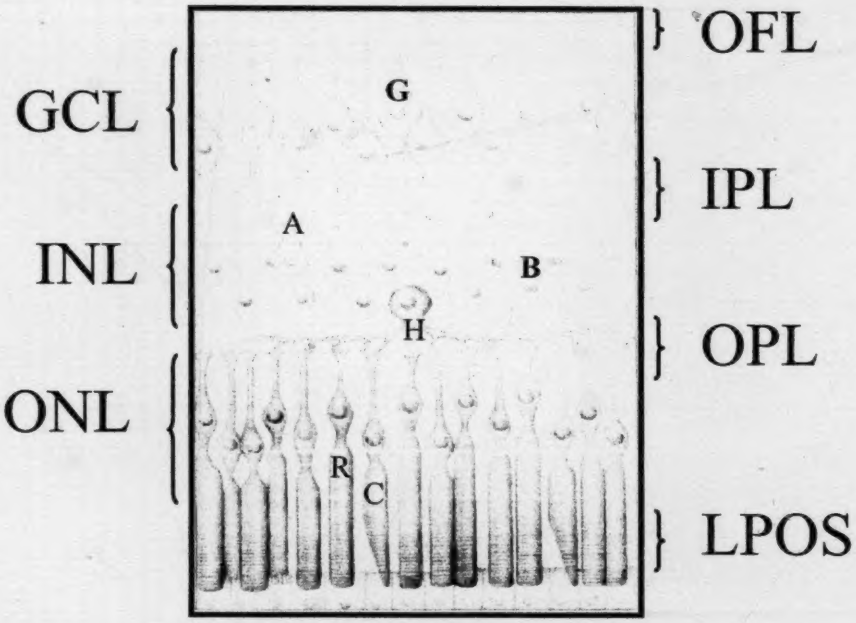

Fig. 1.2. Neural retina cytolology and lamination (modified and reprinted from Neuroscience, Exploring the brain, second edition, M Bear, B Connors & M Paradiso, "The Eye", p286 & p292, Copyright (2001), with permission from Lippincott Williams & Wilkins). The neural retina is divided into three nuclear layers (1-3) and two plexiform layers (4-5): **(1)** the outer nuclear layer (ONL) composed of the cell bodies of photoreceptor cells [cones (C), and rods (R)], **(2)** the inner nuclear layer (INL) composed of the cell bodies of horizontal (H), bipolar (B), amacrine (A) cells and few displaced ganglion (G) cells, and **(3)** the ganglion cell layer (GCL), composed of retinal ganglion cells (RGCs) and displaced amacrine cells. **(4)** The outer plexiform layer (OPL) comprises synapses between photoreceptors, horizontal and bipolar cells, wherea **(5)** the inner plexiform layer (IPL) comprises synapses between bipolar cells, amacrine cells and ganglion cells. The layer of photoreceptor outer segments (LPOS) is the outermost layer of the NR and the optic fiber layer (OFL) is the innermost layer, adjacent to the vitreous humor, and is composed of RGC axons. Müller cells (not shown here) span the neural retina, from the inner to the outer limiting membrane. The inner limiting membrane separates the OFL from the vitreous whereas the outer limiting membrane separates the ONL from the LPOS.

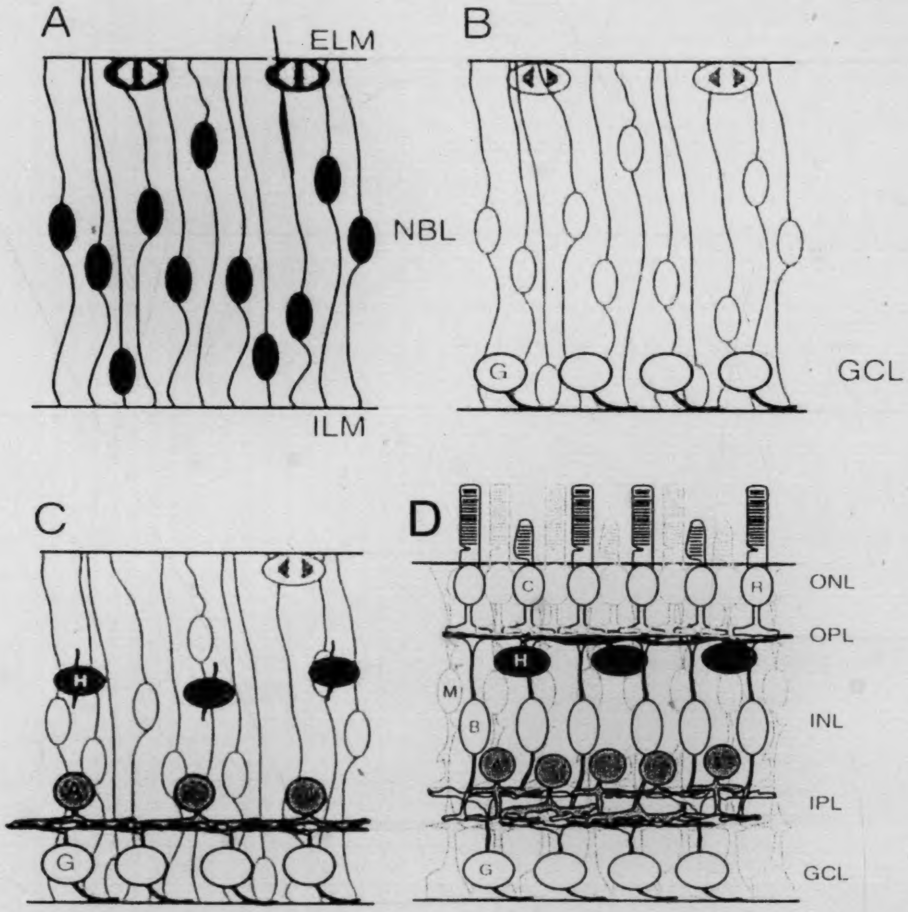

Fig. 1.3. Progressive lamination of the vertebrate neural retina (modified and reprinted from The Visual Neurosciences, Vol. 1, Chapter 6, RO Wong and L Godinho, "Development of the Vertebrate Retina", p78, Copyright (2004), with permission from MIT press). (**A**) As reviewed by Wong and Godinho (2004), cell mitosis (M-phase) occurs in the outmost part of the neural retina, adjacent to the external limiting membrane (ELM). Each newly formed progenitor cell, already attached to the ventricular side at the ELM, extends a process towards the vitreal side which attaches to the inner limiting membrane (ILM). The cell nucleus then migrates back-and-forth between ventricular and vitreal sides during the remaining of the cell cycle. The cell then retracts its vitreal projecting cytoplasmic process and reenters the cell cycle or leaves it. These cells constitute the neuroblastic layer (NBL). (**B**) RGCs are the first retinal cell type to leave the cell cycle and newly postmitotic RGCs (G) are located near the ILM in the ganglion cell layer (GCL) and start extending their axon. (**C**) Amacrine (A) and horizontal (H) cells then differentiate; amacrine cells start to establish connections with RGCs in the inner plexiform layer (IPL). (**D**) The mature retina encompasses three additional differentiated cells types [cones (C), bipolar (B) and Müller cells (M)]. The IPL is itself multilaminated and comprises sublaminas. Abbreviations: IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

detected within the inner nuclear layer of the neural retina outside of this zone (Otteson et al., 2001). Higher vertebrates were also recently shown to possess a CGZ, at least in birds (Fischer and Reh, 2000). In hatched chickens, a CGZ has been described whose progenitor cells proliferate after visual deprivation but not after acute tissue damage (Fischer and Reh, 2000). In adult mammals, progenitor cells are found in the pigmented ciliary margin and have stem cell-like properties *in vitro* (Ahmad et al., 2000; Tropepe et al., 2000).

The eye possesses a blood-ocular barrier that comprises the blood-retinal barrier (BRB) and the blood-aqueous barrier (BAB). In addition, the BRB can be subdivided into the inner and outer BRB (Cunha-Vaz, 1976; Cunha-Vaz, 1979). The inner and outer part of the neural retina is nourished by capillaries encased inside the inner part of the neural retina and by fenestrated capillaries within the choroid, respectively (Hosoya and Tomi, 2005). Tight junctions between retinal endothelial cells and cells of the retinal pigmented epithelium restricts the extravasation of fluid and proteins to the retina and constitute the basis of the inner and outer BRB, respectively (Cunha-Vaz, 1976; Hosoya and Tomi, 2005). In contrast, the retina is not vascularized in the chicken and blood vessels are confined to the pecten oculi outside the retina, in addition to the choroid (Güntürkün, 2000). Tight junctions between endothelial cells within the pecten oculi constitutes a blood-retinal barrier in this species (Gerhardt et al., 1996). In addition, the BAB is constituted by the epithelium and endothelium of the iris and epithelium of ciliary body (Freddo, 2001).

1.1.1.2 Anatomy of the retinal projection axons

RGCs project their axons from the retina to specific targets within the brain; this neural tract is also referred to as the "retinofugal projection" (Mey and Thanos, 2000). The RGC axons from the OFL coalesce at the back of the eye, exit the eye at the optic nerve head and become the optic nerve. Both optic nerves decussate at the optic chiasm. In monocular species such as chicken, all axons decussate (Ramón y Cajal, 1911). Following the optic chiasm, RGC axons form the optic tract and synapse with their targets in the brain. In chickens, RGC axons project chiefly to the optic tectum but also to the thalamus (Fig. 1.4) (Mey and Thanos, 2000). In the mouse, RGC axons project to the superior colliculus, the equivalent to the optic tectum (Sachs et al., 1986), and to the

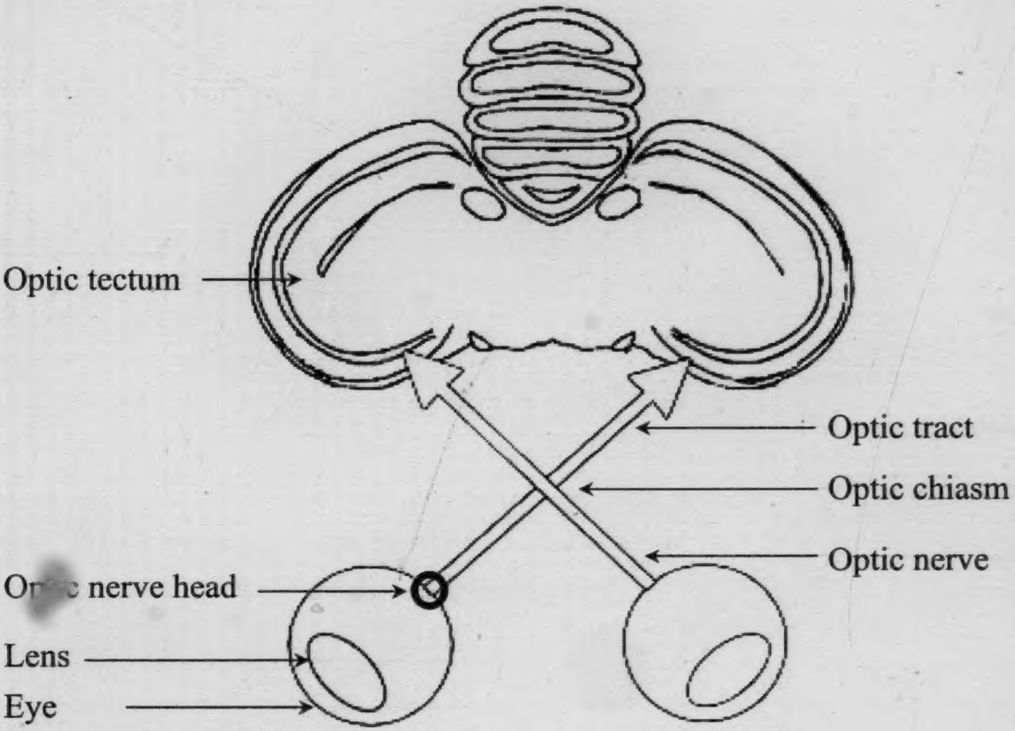
Optic tectum
Optic tract
Optic chiasm
Optic nerve
Optic nerve head
Lens
Eye

Fig. 1.4. The avian retinotectal projection (modified and reprinted from Brain research reviews, volume 32, J Mey & S Thanos, "Development of the visual system of the chick. I. Cell differentiation and histogenesis", p365, Copyright (2000), with permission from Elsevier). RGCs are the projecting neurons of the retina. Their axons bundle together, and can be divided into two topographical structures: the optic nerve before decussation at the optic chiasm, and the optic tract, after decussation. RGC axons synapse chiefly within the optic tectum in chicken.

lateral geniculate nucleus (Godement et al., 1984).

1.1.1.3 Anatomy of the avian optic tectum

The mature chick optic tectum is composed of 6 main strata: (1) the superficial *stratum opticum (SO)*, composed of contralateral RGC axons, (2) the *stratum griseum et fibrosum superficiale* (SGFS), where retinal fibers terminate after leaving the SO, (3) *the stratum griseum centrale* (SGC), composed of ganglion cells that project the main tectal efferent fibers, (4) the *stratum album centrale* (SAC), a plexiform layer composed of fibers such as projecting axons from the SGC ganglion cells, (5,6) and the *stratum griseum periventriculare* (SGP) and *stratum fibrosum periventriculare* (SFP), adjacent to the ependymal layer (Ramón y Cajal, 1911; Huber and Crosby, 1933). These strata are established progressively during development (LaVail and Cowan, 1971a, b) and the early developing optic tectum can be divided into three zones (Gray and Sanes, 1991; Scicolone et al., 1995; Mey and Thanos, 2000): the ventricular zone (VZ), the intermediate (mantle) zone (IZ), and the marginal zone (MZ) (later referred to as the tectal plate (TP) (Fig. 1.5).

1.1.2 Development of the neural retina

The development of the neural retina can be divided into two steps: a first step leading to the formation of the pseudostratified neural retina within the optic cup and a second step leading to the formation of a fully developed multilaminated and functional visual tissue.

1.1.2.1 Early ocular development

The optic pit is the first distinguishable morphological structure of the presumptive neural retina and develops from the prosencephalon (the most proximal brain vesicle). The neural retina is as such part of the central nervous system.

1.1.2.1.1 Early eye development

The neural retina develops from a complex series of events that occur as early as the gastrula stage, soon after the three germ layers (ectoderm, mesoderm, endoderm) are formed (Scott, 2006). Retinal progenitor cells belong to the eye fields within the anterior neural plate of the gastrula. The eye fields evaginate from the neural folds of the forebrain, forming the optic pits, and prolonged evagination results in the elaboration of

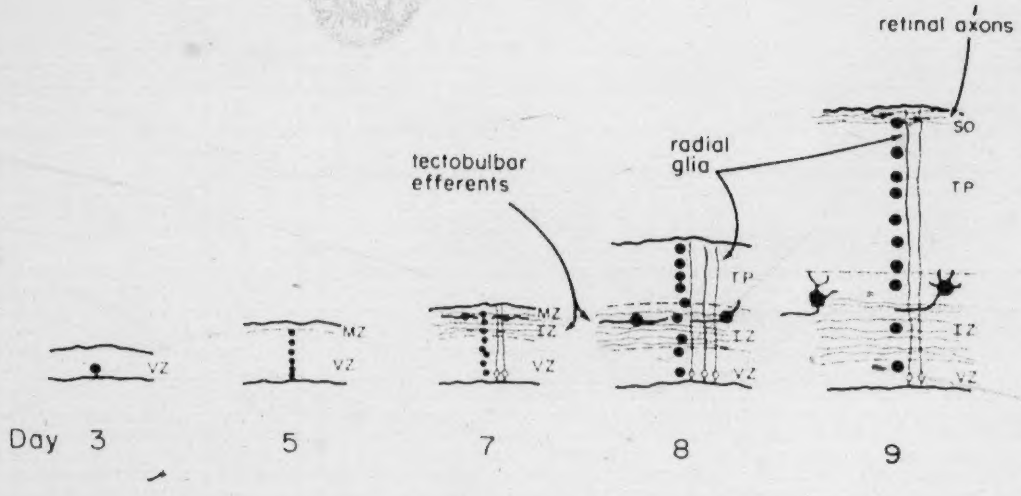

Fig. 1.5. Laminar organization of the developing avian optic tectum (modified and reprinted from Neuron, volume 6, JE Gray and JR Sanes, "Migratory paths and phenotypic choices of clonally related cells in the avian optic tectum", p222, Copyright (1991), with permission from Elsevier). The developing optic tectum can be divided into three zones (Gray and Sanes, 1991; Scicolone et al., 1995; Mey and Thanos, 2000): (**1**) the ventricular zone (VZ), adjacent to the ventricle and containing proliferating progenitor cells, (**2**) the intermediate (mantle) zone (IZ), containing cells migrating from the ventricular zone (black dots), and the circumferential axons of the tectobulbar tract, and (**3**) the marginal zone (MZ) [later referred to as the tectal plate (TP)], located under the pial surface and containing postmitotic cells arranged in radial columns and processes of radial glial cells. The tectum is invaded by retinal axons (starting from E6 and visible here at E9), which remain superficial in the *stratum opticum* (SO).

optic vesicles (Chow and Lang, 2001). The optic vesicles come in close proximity to the surface ectoderm. Factors from the optic vesicle [fibroblast growth factor-8 (FGF-8), bone morphogenetic protein-4 (BMP-4) and BMP-7] then induce the differentiation of the lens from the surface ectoderm (Furuta and Hogan, 1998; Lovicu and Overbeek, 1998; Wawersik et al., 1999; Vogel-Hopker et al., 2000). The newly formed lens placode and the optic vesicle both subsequently invaginate and will form the lens vesicle and the optic cup, respectively (Chow and Lang, 2001).

1.1.2.1.2 Early lens and cornea development

The lens vesicle develops further as a result of continuous stimulation with optic vesicle-derived FGF-8 and BMP-4 (Furuta and Hogan, 1998; Vogel-Hopker et al., 2000). The posterior lens cells differentiate into crystalline-expressing fiber cells, while the anterior cells form the lens epithelium (Chow and Lang, 2001). Posterior fiber cells are regenerated throughout adulthood from cell proliferation at the lens epithelium (Chow and Lang, 2001). Following lens invagination and the formation of the lens vesicle, the remaining surface ectoderm overlying the lens constitutes the presumptive corneal epithelium (Sivak and Sivak, 2000). The epithelium secretes collagen-rich stroma, which will later be invaded by mesenchymal cells (Hay, 1979). Finally the endothelium develops; in embryonic day (E) 7 (of the 21 day incubation period) chick embryos, the stroma is thus delineated by the corneal epithelium and endothelium (Hay, 1979).

1.1.2.1.3 Early retina development

The optic cup is initially composed of two layers. The layer closest to the lens vesicle develops further into the neural retina and the layer farthest from it will form the retinal pigmented epithelium (Chow and Lang, 2001). The surface ectoderm is important for neural retina specification; lens vesicle derived FGF-1 and 2 promote the establishement of neural retina fate while repress that of retinal pigmented epithelium within the optic cup (Guillemot and Cepko, 1992; Hyer et al., 1998; Nguyen and Arnheiter, 2000). The ciliary body develops from the edge of the optic cup (Beebe, 1986) and the aggregating mesenchyme surrounding the optic cup will form the choroid and sclera, while mesenchymal cells migrating into the optic cup will produce the vitreous body (Mey and Thanos, 2000). The edges of the optic cup fuse at the optic (choroid) fissure and will be later traversed by retinal projecting axons (Chow and Lang, 2001).

1.1.2.2 Development of the neural retina

The neural retina develops from a pseudostratified neuroepithelium to an array of laminated nuclear and plexiform layers (Meller and Tetzlaff, 1976). This requires a succession of developmental processes, including cell proliferation, migration, differentiation, neuritogenesis, synaptogenesis and cell death, and entails a complex interplay of various intrinsic and extrinsic factors. The next section will briefly review these main events and focus particularly on the development of RGCs and on the growth factors involved in each process.

1.1.2.2.1 Proliferation

Regulation of cell number is an important process in the developing retina. Transcription factors, growth factors as well as contact mediated signaling appear to be important to regulate proliferation in retinal neuroepithelial cells. Expression of Chx10 transcription factor promotes neural retina progenitor cell proliferation (Burmeister et al., 1996). Delta-Notch mediated juxtracrine signaling has been shown to maintain cells in a proliferative state (Dorsky et al., 1995; Jadhav et al., 2006); cells expressing delta prevents their notch expressing neighboring cells from entering the neuronal differentiation pathway via lateral inhibition (Austin et al., 1995; Henrique et al., 1997; Waid and McLoon, 1998), while they themselves can differentiate as neurons (Dorsky et al., 1997; Henrique et al., 1997). Hormones and growth factors have also been implicated in retinal proliferation. In the developing chick retina, insulin-like factor-1 (IGF-1), insulin, proinsulin, BMP-4, vascular epithelium growth factor (VEGF) are involved (Hernandez-Sanchez et al., 1995; Trousse et al., 2001a; Hashimoto et al., 2006), whereas ciliary neurotrophic factor (CNTF) , epidermal growth factor (EGF) in rats, transforming growth factor-α (TGF- α), FGF-1 and 2, and sonic hedgehog (Shh) are involved in mice and rats (Anchan et al., 1991; Lillien and Cepko, 1992; Jensen and Wallace, 1997; Levine et al., 1997; Wang et al., 2005; Zhang et al., 2005). TGF-β in chicks and rats, on the other hand (Bovolenta et al., 1996; Close et al., 2005), and neurotrophin-3 (NT-3) in chicks (Bovolenta et al., 1996) inhibit mitosis in the neural retina.

Glial cell proliferation is also increased by EGF in rabbits and rats (Scherer and Schnitzer, 1994; Close et al., 2006), by FGF-1 and 2 in rabbits (Scherer and Schnitzer, 1994), by CNTF in mouse (Goureau et al., 2004) and by FGF-9 in rats (Cinaroglu et al., 2005).

1.1.2.2.2 Cell fate determination

In vertebrates, ganglion cells are always the first neurons to leave the cell cycle and bipolar and Müller cells are the last (Young, 1985; Prada et al., 1991). There is, however, a strong overlap between the period during which different cell types exit the cell cycle (Young, 1985; Prada et al., 1991). Instrinsic factors, especially members of the basic loop-helix-loop (bHLH) transcription factors, play an important role in cell fate determination (Cepko, 1999; Cayouette et al., 2006). Extrinsic growth factors such as CNTF, taurine, and FGF in rats and TGF-β in *Xenopus* have also been shown to promote the production of one cell type at the expense of another (Altshuler et al., 1993; Lillien, 1995; Ezzeddine et al., 1997; McFarlane et al., 1998).

1.1.2.2.3 Differentiation

After cell cycle exit, retinal cells undergo maturation, whereby they acquire their morphological and functional traits. In vertebrates, retinal neurogenesis is initiated by RGC production (Esteve and Bovolenta, 2006). The onset of RGC neurogenesis occurs in a specific location that differs among vertebrates and spreads topographically in a species-specific pattern (Esteve and Bovolenta, 2006). For instance in chicks, the first RGCs are formed in an area central to the retina and dorsotemporal to the choroid fissure and RGC differentiation spreads according to a centrofugal gradient (Kahn, 1973, 1974; Prada et al., 1991). The area where neurogenesis is initiated is also a site of FGF expression and FGF signaling triggers the initiation of ganglion cell neurogenesis, at least in fish and chicks (Martinez-Morales et al., 2005; Esteve and Bovolenta, 2006).

Postmitotic RGC precursors first acquire a state of competence [i.e. the ability to respond to specific cue(s) (Livesey and Cepko, 2001)] defined by the transient expression of the proneural bHLH transcription factor ath5 (Kay et al., 2001; Yang et al., 2003). Ath5, in turn, initiates the transcription of molecules, such as Pou4f2 (formerly Brn3.2 or Brn3b), a POU domain transcription factor (Liu et al., 2001; Wang et al., 2001; Yang et al., 2003), important for the proper maturation of RGCs (Erkman et al., 1996; Gan et al., 1996; Xiang, 1998; Gan et al., 1999). Pou4f2 likely controls RGC differentiation via other genes encoding transcription factors and proteins required for neuron integrity and function, as well as proteins involved in signal transduction (Mu et al., 2004). Pou4f2 also appears to be important for axon growth and cell polarization (Wang et al., 2000), axon guidance (Erkman et al., 2000) and cell survival (Xiang, 1998; Gan et al., 1999).

Growth factors have been shown to alter the differentiation of retinal cells. FGF-2 (in rats and chicks) (Zhao and Barnstable, 1996; Desire et al., 1998b), NT-3 (Bovolenta et al., 1996), and FGF-1 (in chicks) (Guillemot and Cepko, 1992) induce an increase in the number of RGCs while VEGF inhibits RGC production (in chicks) (Hashimoto et al., 2006). Similarly FGF-2 and TGF-α in a human retinal precursor cell line (Ezeonu et al., 2000; Ezeonu et al., 2003), FGF-1 and FGF-2 in chicks (Desire et al., 1998a), CNTF (in chicks) (Fuhrmann et al., 1995), and FGF-2 (in rats) (Hicks and Courtois, 1992) promote photoreceptor differentiation whereas EGF inhibits it in rats (Ahmad et al., 1998). CNTF promotes Müller glial cell genesis in the postnatal mouse retina via a STAT3 and ERK activation (Goureau et al., 2004). IGF-1, insulin, and NT-3 promote the production of G4 (a neuroglial cell adhesion molecule, Ng-CAM) expressing neurons in chicks (de la Rosa et al., 1994; Hernandez-Sanchez et al., 1995; Frade et al., 1996b), and this IGF-1 role is associated with the upregulation of α6 integrin subunit of the laminin receptor (Frade et al., 1996b). The differentiation of these cells was mostly determined by measuring the appearance of a specific cell type labeled with a cell marker and only CNTF and FGF-2 have been shown to be *bona fide* differentiation factors (Hicks and Courtois, 1992; Fuhrmann et al., 1995). The roles of most of these factors in differentiation are therefore speculative, especially as apoptosis and proliferation can alter the number of differentiated cells.

1.1.2.2.4 Cell migration

In vertebrates, cells leave the cell cycle at the ventricular surface of the retina and subsequently migrate to their final position. The nucleus of migrating cells translocates to its final destination while the cell soma remains attached to both the scleral (ventricular) and vitreal (pial) surface of the retina using their process endfeet (McLoon and Barnes, 1989; Dunlop, 1990; Snow and Robson, 1994, 1995). These processes retract during or after the migration is complete (Prada et al., 1981; Snow and Robson, 1995) (Fig. 1.3). Thus, retinal migration does not involve radial glia (Snow and Robson, 1994, 1995), whose processes act as guides for migrating neurons in other developing nervous tissues, such as the optic tectum (Gray and Sanes, 1991, 1992; Snow and Robson, 1995).

The basal lamina of the inner limiting membrane is important for cell migration, as it enables the anchorage of process endfeed to the vitreal surface (Mey and Thanos, 2000). This anchorage might be mediated by the laminin receptor subunit, β1 integrin, as its immunoneutralization disrupts proper ganglion cell migration (Cann et al., 1996).

1.1.2.2.5 Neuritogenesis

1.1.2.2.5.1 Axon growth

Axonogenesis requires the synthesis of axonal proteins and lipids, and their insertion into the growing process at the growth cone, the leading tip of the growing axon, and along the axon (Goldberg, 2003). Very little is known about neurite outgrowth from retinal cells other than RGCs. An axon is generated from RGC vitreal endfeet or from RGC soma (Nishimura, 1980; Prada et al., 1981; Watanabe et al., 1991); RGC axonogenesis is thus polarized. This polarized growth is influenced by Müller cells, as axons can grow on Müller cell endfeet but they are inhibited by their somata (Bauch et al., 1998; Stier and Schlosshauer, 1998, 1999). Conversely, dendrites can grow on Müller cell somata located in the outer retina (Bauch et al., 1998).

Intrinsic and extrinsic mechanisms regulate axon growth (Goldberg, 2003). RGC Pou4f2 transcription factor promotes axon growth and fasciculation (Gan et al., 1999; Wang et al., 2000). Another POU domain transcription factor, Pou4f3, also induces RGC neurite outgrowth independently of Pou4f2 (Wang et al., 2002). Interestingly, RGCs lose their ability to extend their axons over time, as older developing RGCs do not elongate axons as fast as young RGCs in similar environments (Cohen et al., 1986; Hall et al., 1987; Goldberg et al., 2002a). This irreversible loss in intrinsic ability for axon growth is induced by membrane-associated extrinsic signals emanating from amacrine cells, at least in rodents (Goldberg et al., 2002a).

Ganglion cells do not extend an axon by default, as Bcl-2 overexpressing RGCs do not project neurites (Goldberg et al., 2002b). Extrinsic factors thus appear to be crucial for RGC axon growth; extracellular matrix (ECM) and cell adhesion molecules, as well as growth factors have all been shown to promote axon elongation in developing ganglion cells. RGC axons elongate on laminin, collagen and vitronectin ECM substrates

(Thompson and Pelto, 1982; Cohen et al., 1987; Halfter et al., 1987; Pierce et al., 1988; Carri et al., 1992; Martinez-Morales et al., 1995) but not on agrin (Halfter et al., 1997). Moreover, the cell adhesion molecule β1 integrin promotes collagen and laminin mediated axon outgrowth (Drazba and Lemmon, 1990; Carri et al., 1992; Lilienbaum et al., 1995; Sakaguchi and Radke, 1996) while neural-cell adhesion molecule (N-CAM), L1, and N-cadherin promote axon outgrowth (Drazba and Lemmon, 1990; Riehl et al., 1996) on glial endfeet (Drazba and Lemmon, 1990).

Brain-derived neurotrophic factor (BDNF) and NT-4 in embryonic rats (Avwenagha et al., 2003) and BDNF, CNTF, and leukaemia inhibitory factor (LIF) in early postnatal rats have also been shown to stimulate RGC axon growth (Jo et al., 1999; de Ary-Pires and Linden, 2000; Goldberg et al., 2002b). The actions of BDNF and CNTF are enhanced by cAMP accumulation and by membrane depolarization (de Ary-Pires and Linden, 2000; Goldberg et al., 2002b). In addition, apolipoprotein E derived from rat cortical glial cells (Hayashi et al., 2004) and receptors for protein tyrosine phosphatase (RPTP) such as CRYPα and PTP–δ in chicks and *Xenopus* (Ledig et al., 1999; Johnson et al., 2001) and Shh in chicks (Trousse et al., 2001b), have also been shown to modulate RGC axon growth.

1.1.2.2.5.2 Dendrite growth

In RGCs, dendritogenesis occurs after axon growth is initiated (Thanos and Mey, 2001). RGC dendrite growth is under the control of adhesion molecules, such as N-cadherins (Riehl et al., 1996) and β1 integrin (Lilienbaum et al., 1995). It is also dependant upon neighbouring cell density and contact (Troilo et al., 1996; Sernagor et al., 2001; Lin et al., 2004), trophic factors and electrical activity. In RGCs, retina-derived BDNF inhibits dendritic arborization while target-derived BDNF promotes the arborization of dendrites in the retina and the arborization of the axon in the target in *Xenopus* (Lom and Cohen-Cory, 1999; Lom et al., 2002). In addition in RGCs, immature spontaneous bursts of electrical activity promote RGC dentritic growth in turtles (Sernagor and Grzywacz, 1996; Mehta and Sernagor, 2006). After the elaboration of specific branching and arbors, RGC dendrites are remodeled / pruned (Dann et al., 1987; Wong, 1990; Yamasaki and Ramoa, 1993).

During the dendritic growth phase, RGC dendrites elongate within the inner plexiform layer, which later becomes stratified in laminas upon pruning of the dendritic tree (Sernagor et al., 2001). The inner plexiform layer stratification is controlled by neurotransmission, as inhibition of glutamate release from neighboring cells delays sublamina formation (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995). Dendritic remodeling is, however, not influenced by electrival activity derived from RGCs, at least in cats (Wong et al., 1991).

1.1.2.2.6 Programmed cell death

Neurons are generated in excess during nervous system development and an estimated 50% of developing retinal cells die (Voyvodic et al., 1995). In chick embryos, two waves of programmed cell death (also commonly referred to "apoptosis") have been reported in the developing retina. The first wave (E5-E8, peaks at E6) occurs during the period of cell proliferation, migration and differentiation and is thought to affect neuroblasts or early postmitotic cells as a way to regulate retinal cell number and/or cell fitness (Rager, 1980; Frade et al., 1997; de la Rosa and de Pablo, 2000). The second wave (E9-E14, peaks at E12) corresponds to the well described period of ganglion cell death following target innervation (Rager and Rager, 1978; Hughes and McLoon, 1979; Frade et al., 1997) and would selectively affect RGCs with inappropriate connections and/or supernumerary axons (Rager and Rager, 1978; O'Leary et al., 1986). This latter wave has been reported in all species studied (Rager and Rager, 1978; Young, 1984; Isenmann et al., 2003) and would affect 40-50% of ganglion cells (Rager, 1980; Perry et al., 1983; Voyvodic et al., 1995). In the chick, the first wave is preceded by a period of cell death in retinal areas to be crossed by growing ganglion cell axons and could thus create space for the growth of RGC axons (Cuadros and Rios, 1988; Martin-Partido et al., 1988). In addition to RGC, cell death also occurs in precursor and postmitotic oligodendrocytes in the developing optic nerve head, at least in rats (Barres et al., 1992).

During development, survival of the ganglion cells is regulated by various families of molecules. Bcl-2 family members regulate programmed cell death (Martinou et al., 1994; Mosinger Ogilvie et al., 1998; Cellerino et al., 1999; Cory et al., 2003) while caspases, a family of cysteine proteases, execute it (Kuida et al., 1996; Thornberry and Lazebnik, 1998). In addition, caspase-independent proapoptotic molecules, e.g. calpain, and AIF-1 (apoptosis inducing factor-1) also promote cell death in the developing retina (Azarian and Williams, 1995; Harvey et al., 2006b; Sanders et al., 2006).

Many growth factors regulate cell death or cell survival in the developing retina. For instance in the chick embryo, FGF-2, BDNF, insulin, and GH have all been shown to prevent retinal cell death (Frade et al., 1997; Desire et al., 1998b; Diaz et al., 1999; Diaz et al., 2000; Sanders et al., 2005). Conversely, TGF-β, BMP-4, FGF-2 and nerve growth factor (NGF) induce retinal cell death during the same developmental period (Frade et al., 1996a; Yokoyama et al., 1997; Frade and Barde, 1998; Dunker et al., 2001; Trousse et al., 2001a; Schuster et al., 2002; Franke et al., 2006). During the second wave of programmed cell death, TGF-β and BMP-4, FGF-2 are proapoptotic, whereas BDNF and NGF are antiapoptotic (Frade et al., 1997; Desire et al., 1998b; Karlsson et al., 2001; Franke et al., 2006).

In rodents, TGF-β and BMP–4 are proapoptotic, while insulin, proinsulin, IGF-1, BDNF, NT4/5, CNTF, LIF, FGF-2, and TGF-α are antiapoptotic, respectively, in developing embryonic (Frade and Barde, 1999; Beier et al., 2006; Valenciano et al., 2006) and postnatal retinas (Politi et al., 2001; Cusato et al., 2002; Dunker and Krieglstein, 2003; Duenker et al., 2005; Beier et al., 2006; Franke et al., 2006).

Very few studies, so far, have clearly established the requirement of target-derived trophic factors to promote the survival of RGCs. While BDNF has been reported to be present in both the tectum and retina of the chick visual system, only retinal BDNF is thought to induce cell survival (Herzog and von Bartheld, 1998). In the neonate hamster, BDNF injected into the colliculus promotes also RGC survival and this action might act directly on retinal target cells, as BDNF is transported to the retina following injection (Ma et al., 1998).

1.1.2.3 Development of the avian optic tectum

RGC axons project mainly to the contralateral optic tectum in the chicken (Mey and Thanos, 2000). Interestingly, the retina and its target develop simultaneously but independently until they are connected via the RGC axons (Cowan et al., 1968; Kelly and Cowan, 1972; Hughes and McLoon, 1979; Vanselow et al., 1990; Yamagata and Sanes, 1995).

1.1.2.3.1 Cell proliferation and differentiation

In the chick embryo, cell mitosis occurs mostly between E3 and E6 and is complete by E12 (Mey and Thanos, 2000). It is first confined to the ventricular zone (Fig. 1.5), but after E5 it is encountered in all developing strata (Cowan et al., 1968). Cells start to leave the cell cycle at E4-E6 and the first cells to differentiate are the ganglion cells of the prospective SGC (also referred to as multipolar cells) (LaVail and Cowan, 1971a; Domesick and Morest, 1977). Glial cells and neurons emanate from a common progenitor (Galileo et al., 1990). Early on, ganglion cells project axons, referred to as the tectobulbar tract, that contribute to the circumferential fibers of the superficial marginal zone and will later contribute to the SAC (Goldberg, 1974; Kroger and Schwarz, 1990; Gray and Sanes, 1991). G4 and N-cadherin promote tectobulbar axon fasciculation and T61 promotes their growth (Kroger and Schwarz, 1990; Treubert-Zimmermann et al., 2002). As cell migration progresses, the tectobulbar tract becomes progressively deeper within the tectum and never actually makes contact with the innervating fibers of the retina (Goldberg, 1974). Overall, the optic tectum develops according to a rostro-ventrolateral to caudo-dorsomedial gradient (Cowan et al., 1968; LaVail and Cowan, 1971a).

1.1.2.3.2 Cell migration

Tectal cells migrate using two mechanisms. The perikaryon of some cells translocates while they are attached to both the pial and ventricular surfaces via processes and endfeet (Puelles and Bendala, 1978; Snow and Robson, 1995). Cells of the optic tectum also migrate using radial glia processes as guides (Gray and Sanes, 1991, 1992; Snow and Robson, 1995). Tangential migration along tectobulbar fibers of the marginal zone is also observed (Gray and Sanes, 1991; Mey and Thanos, 2000). β1-integrin is involved in this process, as impairing its translation disrupts tectal cell migration (Galileo et al., 1992).

1.1.2.3.3 Tectal innervation

In chicken, the first RGC axons project to the optic tectum starting at E6 and the optic tectum is entirely covered with retinal axons, now forming the SO, by E12 (Goldberg, 1974; Rager and von Oeynhausen, 1979). RGC axons, however, start penetrating the SGFS and branch within it at E9 (Crossland et al., 1975; Rager and von Oeynhausen, 1979; Thanos and Bonhoeffer, 1987) but (according to most researchers) mature synaptic contacts are not observed before E10 (Cantino and Sisto Daneo, 1973; Rager, 1976; Panzica and Panzica, 1981). N-cadherin, neurotrophins, and glycoconjugates are involved in the establishment of RGC connections within the tectum (Inoue and Sanes, 1997). Axons do not reach their terminal location or "terminal zone" as they reach the tectum (Nakamura and O'Leary, 1989). Instead, targeting errors are iniatially made but later corrected (Nakamura and O'Leary, 1989; Nakamura et al., 1990). Ephrins and their retinal axon receptors Eph are essential in directing axons to their final destination (McLaughlin and O'Leary, 2005).

The development of the neural retina and of the optic tectum of the visual system consists of the establishment of functional multilaminated tissues. This development comprises a series of events and each event requires the interplay of many molecules including growth factors. The involvement of GH in the development of the neural retina or the optic tectum had rarely been considered before the start of the present work. The next section will thus provide some background on GH and establish that GH is a putative key growth factor in the development of the neural retina, of retinal projecting neurons and possibly of their target, the optic tectum.

1.2 Growth Hormone

GH was first discovered in extracts of the bovine pituitary gland (Evans and Long, 1921) and was named because of its ability to promote growth in bioassay paradigms [reviewed by (Kopchick, 2003)]. Since then, it has been found in pituitary glands of species from all vertebrate groups. GH-like molecules are also present in the nervous system of invertebrates lacking pituitary glands [e.g. (Swinnen et al., 1990)]. It is therefore an ancient molecule that might have had ancestral roles as a local growth factor and acquired *endocrine* roles with the evolution of the vertebrate *endocrine* system (Harvey et al., 1993). *Endocrine, autocrine or paracrine* actions of GH in the central nervous system have been well established (Harvey and Hull, 2003), but the possible involvement of GH in ocular development or vision has rarely been considered. Relevant literature in the GH field is therefore the focus of this review

1.2.1 GH: Synthesis, storage and release

1.2.1.1 GH transcription and translation

GH belongs to the GH / prolactin (PRL) family of hormones and is derived from a common ancestral proto-GH/PRL gene (Niall et al., 1971; Bewley et al., 1972; Miller and Eberhardt, 1983) from which GH and PRL diverged from gene duplication more than 350 million years ago (Niall et al., 1971; Miller and Eberhardt, 1983). In human, the *GH* gene is part of a gene cluster composed of four genes encoding two GH isohormones [GH isohormones differ in their primary structure (Baumann, 1991)], human GH-N (hGH, N for Normal), and hGH-V (V for Variant), and two placental lactogens (PL) PL-A and PL-B. A non-coding pseudogene PL-L is also part of this gene cluster (Owerbach et al., 1980; Harper et al., 1982; Kidd and Saunders, 1982; Hirt et al., 1987). The hGH-N and –V genes are predominantly expressed in the anterior pituitary and in the placenta, respectively (Frankenne et al., 1987; Chen et al., 1989). Other tetrapod vertebrate species, however, encompass one single *GH* gene (Shaw et al., 1991).

The *GH* gene is composed of five exons (1-5) interspaced by four introns (A-D) and its transcription is under the control of a TATA box containing promoter (DeNoto et al., 1981) (Fig. 1.6). The coding regions of mammals, including rodents (Seeburg et al., 1977; Barta et al., 1981; Page et al., 1981; Linzer and Talamantes, 1985) are highly

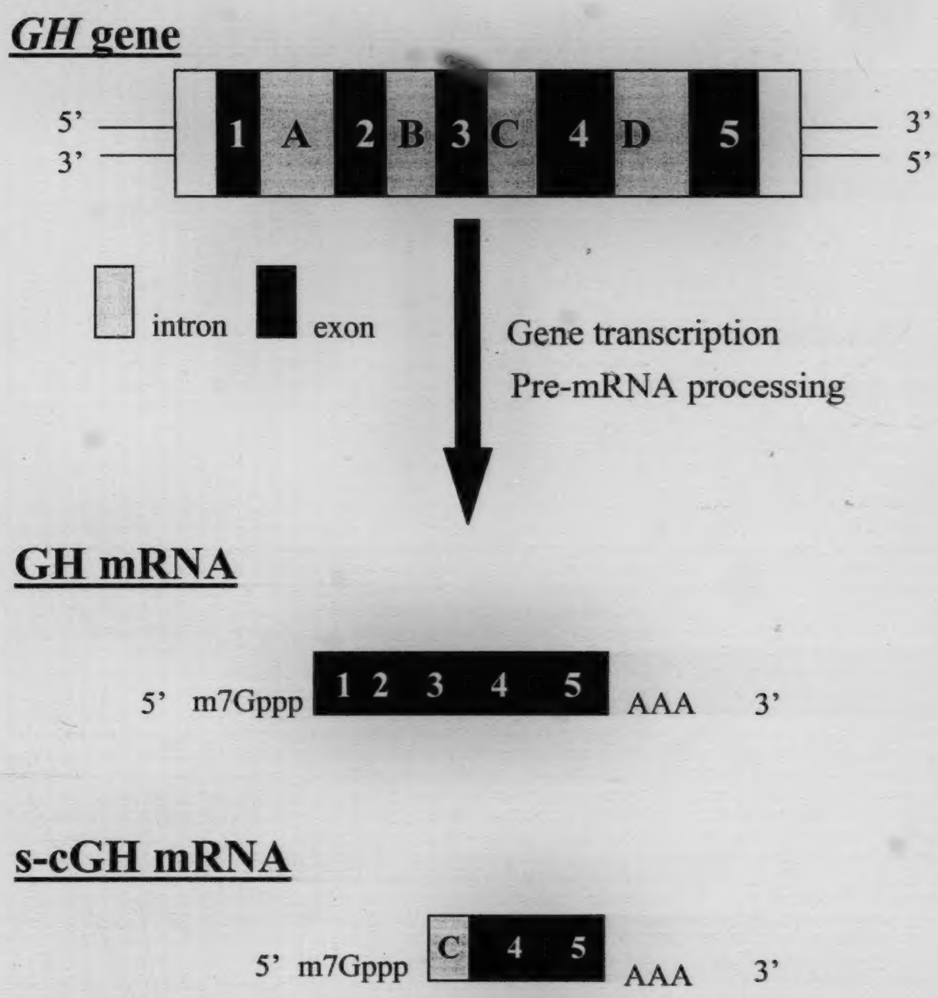
GH gene
5'
3'
1
A
2
B
3
C
4
D
5
3'
5'
intron
exon
Gene transcription
Pre-mRNA processing
GH mRNA
5' m7Gppp
1 2 3 4 5
AAA 3'
s-cGH mRNA
5' m7Gppp
C 4 5
AAA 3'

Fig. 1.6. ***GH* gene and two transcribed mRNAs**. The *GH* gene is composed of 5 exons (1-5) and 4 introns (A-D). Full length GH mRNA is composed of 5 spliced exons (DeNoto et al., 1981), whereas small chicken GH (s-cGH) mRNA sequence encompasses part of the intron C, exon 4 and exon 5 (Takeuchi et al., 2001).

homologous but vary from those in birds (Souza et al., 1984; Tanaka et al., 1992; Harvey, 1995). Polymorphism of the *GH* gene has been reported in various species, including mammals (Chakravarti et al., 1984; Kunieda et al., 1989), birds (Mou et al., 1995) and fish (Forbes et al., 1994; Park et al., 1995).

In the pituitary, the regulation of GH synthesis and release is complex and requires the interplay of many molecules. In somatotrophs, Pit-1 (Pituitary specific transcription factor-1), a POU domain protein, appears to be essential for the initiation of *GH* gene transcription (Nelson et al., 1988; Fox et al., 1990; Pfaffle et al., 1992). Hypothalamic hormones are acknowledged regulators of pituitary function. GH synthesis is stimulated by a growth hormone-releasing hormone (GHRH) and GH release is regulated by both GHRH and somatostatin [also referred to as somatotropin-releasing inhibitory factor (SRIF)] (Gick et al., 1984; Barinaga et al., 1985; Fukata et al., 1985; Vasilatos-Younken et al., 1992). In addition, ghrelin has recently been shown to directly induce GH release from chicken and mammalian pituitary cells (Kojima et al., 1999; Yamazaki et al., 2002; Baudet and Harvey, 2003).

Upon GH gene transcription, a primary transcript is produced. As first described by DeNoto (1981), pre-mRNA processing leads to a 5' UTR (untranslated region) / exons 1 to 5 / 3'UTR containing mRNA (Fig. 1.6). However, pre-mRNA processing can also lead to alternatively spliced variants (Table 1.1). In addition in chicken, a putative alternate promotor controls the transcription of a transcript with a severely truncated 5' end that retains the 3' end of intron C, exon 4 and exon 5 (Fig. 1.6) (Takeuchi et al., 2001).

The 5' end of GH mRNA encodes a N-terminal hydrophobic signal peptide that directs the mature transcript to be translated within the endoplasmic reticulum of the secretory pathway (Martial et al., 1979; Miller et al., 1980). The signal peptide is cotranslationaly cleaved from the pre-hormone (Lingappa et al., 1977) and a mature (monomer) 191 residue, 22,000 Dalton (22K) GH is thus synthesized [e.g. in chicken (Souza et al., 1984; Lamb et al., 1988)].

Monomer GH contains four cysteine residues that contribute to the formation of two intramolecular disulfide bridges (Li and Dixon, 1971; Niall, 1971; Niall et al., 1971). Its three dimensional structure has been characterized by X-ray crystallography; GH is

Table 1.1. GH isohormones

species	Tissue	Deletion/addition	#AA	Name & Putative MW	reference
Human	Pituitary	-45bp at 5'end of exon 3	176*	hGH[1]-N 20K	(DeNoto et al., 1981)
Rat	Pituitary	-45bp at 5'end of exon 3	176*	rGH[2]-N 20K	(Howland et al., 1987)
Human	Placenta	-45bp at 5'end of exon 3	176*	hGH-V 20K	(Boguszewski et al., 1998)
Human	Placenta	-4bp at 3' end of exon 4 Shift in reading frame	219	hGH-V3 24K	(Boguszewski et al., 1998)
Human	Testis	-4bp at 3' end of exon 4 Shift in reading frame	219	hGH-Vδ4 25K	(Untergasser et al., 1998)
Human	Pituitary + tumor	- exon 3	151*	hGH-N 17.5K	(Lecomte et al., 1987) (Palmetshofer et al., 1995)
Human	Pituirary	- exon 3 & 4	96*		(Palmetshofer et al., 1995)
Human	Pituirary	- exon 2, 3 & 4	42*		(Palmetshofer et al., 1995)
Human	Placenta	+ Intron D Shift in reading frame	230	hGH-V2 26K	(Cooke et al., 1988)
Human	Testis	+ Intron D Shift in reading frame	230	hGH-V2 26K	(Untergasser et al., 1997)
Bovine	Pituitary	+ Intron D Shift in reading frame	233	bGH[3]-N 27K	(Hampson and Rottman, 1987)
Chicken	Retina	- exon 1-3; + 3' end of intron C	140	scGH[4] 16.5K	(Takeuchi et al., 2001)

* deduced from publication and size of GH = 191 amino acids

composed of four antiparallel alpha-helices (Abdel-Meguid et al., 1987; de Vos et al., 1992) (Fig. 1.7A). GH tertiary structure is essential for its bioactivity, since it constitutes the basis for binding to and activating GH receptor (GHR) (Waters et al., 2006) .

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[1] hGH: human GH
[2] rGH: rat GH
[3] bGH: bovine GH
[4] scGH: small chicken GH

1.2.1.2 GH posttranslational heterogeneity.

The newly translated 22K GH may undergo numerous posttranslational modifications, including deamidation (Lewis et al., 1979; Lewis et al., 1981), O-linked glycosylation (Sinha and Lewis, 1986; Berghman et al., 1987; Sinha and Jacobsen, 1987), phosphorylation (Liberti et al., 1985; Liberti and Joshi, 1986; Aramburo et al., 1989), N-terminal acylation (Lewis et al., 1979), dimerization / oligomerization and proteolytic cleavage (Baumann, 1999).

1.2.1.2.1 Dimerization / oligomerization

The 22K monomer has been reported to aggregate to form homodimeric to homopentameric isovariants (Stolar et al., 1984; Houston and Goddard, 1988; Aramburo et al., 1990). The GH homodimer, a well-described oligomer, results from either noncovalent interactions (Chapman et al., 1981; Brostedt and Roos, 1989) or from intermolecular disulfide bridges (Singh et al., 1974; Benveniste et al., 1975; Lewis et al., 1977) and forms 44-45K GH isovariants. Some dimeric GHs have been reported to be particularly stable to denaturing and reducing conditions (Aramburo et al., 1990; Grigorian et al., 2005).

1.2.1.2.2 Proteolytic cleavage and submonomer GH

Submonomer GH isovariants result, at least partly, from native or artifactual proteolytic digestion at one of two cleavage sites (residues 43-44 and / or residues 134-146) (Singh et al., 1974; Singh et al., 1983; Nicoll et al., 1986; Baumann, 1991). Thus, a 5K hGH 1-43 and its 17K hGH44-191 counterpart are present in pituitary extracts and serum, respectively (Singh et al., 1983; Sinha and Jacobsen, 1994). Proteolytic cleavage does not, however, necessarily leads to the *de novo* production of submonomer GH. The cleavage of 22K GH can result in the production of a 24K moiety composed of two cleaved fragments covalently linked by the disulfide bridge of the large loop and although the mass of the cleaved GH isoform remains the same, it appears to be 2K larger on electrophoretic gels (Maciag et al., 1980). However, upon its reduction with an

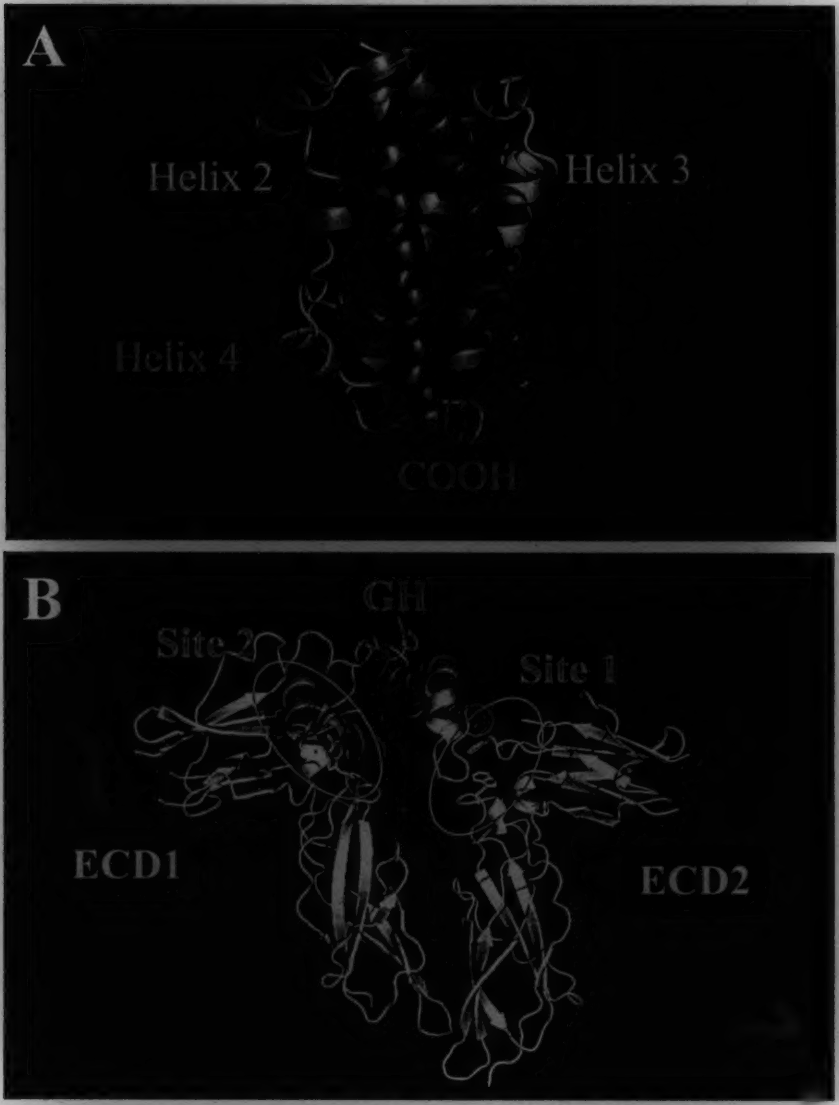
A
Helix 2
Helix 3
Helix 4
COOH
B
GH
Site 2
Site 1
ECD1
ECD2

Fig. 1.7. Tertiary structure of GH and GH receptor (GHR). (**A**) Human GH tertiary structure (modified from Sundstrom et al., 1996) is composed of 4 helices between the N- (NH2) and the C- (COOH) terminus. (**B**) GH [represented here after 90° rotation compared to (A)] binds the extracellular domain (ECD) of two GHRs at GH's site 1 and site 2 (circled in purple).

endogenous protease, two putative 8K and 14K moieties are generated (Maciag et al., 1980). These GH isovariants might occur *de novo*, as they can be produced following the incubation of the 22K GH with liver membranes (an acknowledged GH target tissue) (Schepper et al., 1984).

A 15K GH isoform has also been reported in chicken pituitaries under reducing and non-reducing conditions (Aramburo et al., 1990; Aramburo et al., 2000). Although it is not yet known how this submonomer is synthesized *de novo*, a 15K C-terminus (1-133) moiety is produced from thrombin mediated proteolytic digestion and subsequent reduction of the monomer GH (Aramburo et al., 2001). Moreover, the concentration of this variant, relative to all other GH isoforms, is highest during late embryogenesis (Aramburo et al., 2000). Other submonomers are present in chicken tissues. A 14K and a predominant 17K have been reported in lymphoid tissues and testis (Luna et al., 2004; Luna et al., 2005). 13K and 18K are also present in pituitary tissues under reducing conditions (Aramburo et al., 2000).

GH thus occurs as multiple isoforms and variants, most of which have characterized biological activity. This is of interest, as only monomer GH is likely to be the endogenous ligand.

1.2.2 Growth hormone receptor

The GHR was cloned in the late 1980s from human and rabbit liver (Leung et al., 1987). The chicken *GHR* gene was cloned in the early 1990s and encodes a 608 residue protein with 58% homology with rat GHR and 50% homology with human GHR sequences (Burnside et al., 1991).

1.2.2.1 Growth hormone receptor: characteristics

1.2.2.1.1 Member of the cytokine superfamily

The membrane bound GHR belongs to the class I cytokine / hematopoietic receptor superfamily (Bazan, 1989; Cosman et al., 1990; Zhu et al., 2001). This family comprises 15 members, including the receptors for prolactin, CNTF and LIF (Herrington and Carter-Su, 2001; Zhu et al., 2001). These members are glycoproteins with an extracellular binding domain, a single transmembrane domain and an intracellular domain mediating signal transduction (Zhu et al., 2001).

A circulating and / or tissue GH binding protein (GHBP) shares with GHR its extracellular GH binding domain (Leung et al., 1987) and thus binds GH with high affinity (Ymer and Herington, 1985). GHBP in some species is derived from membrane bound GHR by proteolysis (McGuffin et al., 1976; Trivedi and Daughaday, 1988; Sotiropoulos et al., 1993; Martini et al., 1997; Vleurick et al., 1999) and thus corresponds to the extracellular / binding domain of GHR. GHBP can also be produced from alternative splicing of the GHR mRNA in rats (Baumbach et al., 1989), mice (Edens et al., 1994), guinea pigs (Hull et al., 1996), rhesus macaque (Martini et al., 1997) and chicken (Lau et al., 2006).

The intracellular domain of the GHR binds constitutively to Janus kinase 2 (JAK2) (VanderKuur et al., 1994), which is a tyrosine kinase and the most important signal transduction molecule in GH signaling (Argetsinger et al., 1993; Silva et al., 1993; Zhu et al., 2001). Upon transphosphorylation / activation, JAK2 phosphorylates STAT (signal transducers and activators of transcription). GH induced expression of SOCS (suppressors of cytokine signaling) serves as an intracellular negative feedback loop (Adams et al., 1998), as it inhibits GHR signaling to STAT (Naka et al., 1997; Ram and Waxman, 1999).

1.2.2.1.2 GHR dimerization

One GH molecule binds to two GHRs leading to GHR homodimerization (Cunningham et al., 1991; de Vos et al., 1992). One single GH molecule encompasses two structurally unrelated binding sites (a high affinity "site 1" and low affinity "site 2") that mediate the sequential binding of GH to the GHR homodimer (Fig. 1.7B) (Cunningham et al., 1991; Fuh et al., 1992). The high affinity binding between GH site 1 and one GHR molecule favors the binding of a second GHR at the low affinity site 2; a heterotrimeric complex is thus formed (Cunningham et al., 1991; Fuh et al., 1992). Whilst different residues of the GH molecule contribute to binding to the GHR dimer, identical residues on both GHRs contribute to binding to GH (Fig. 1.8) (de Vos et al., 1992). Until recently, it was thought that GH-induced GHR dimerization was promoting receptor activation (Fuh et al., 1992; Behncken et al., 2000). Recent evidence, however, strongly suggests that the GHR exists as an inactive predimerized complex (Brown et al., 2005). According to Brown and coworkers (2005), GH site 1 would bind to the first

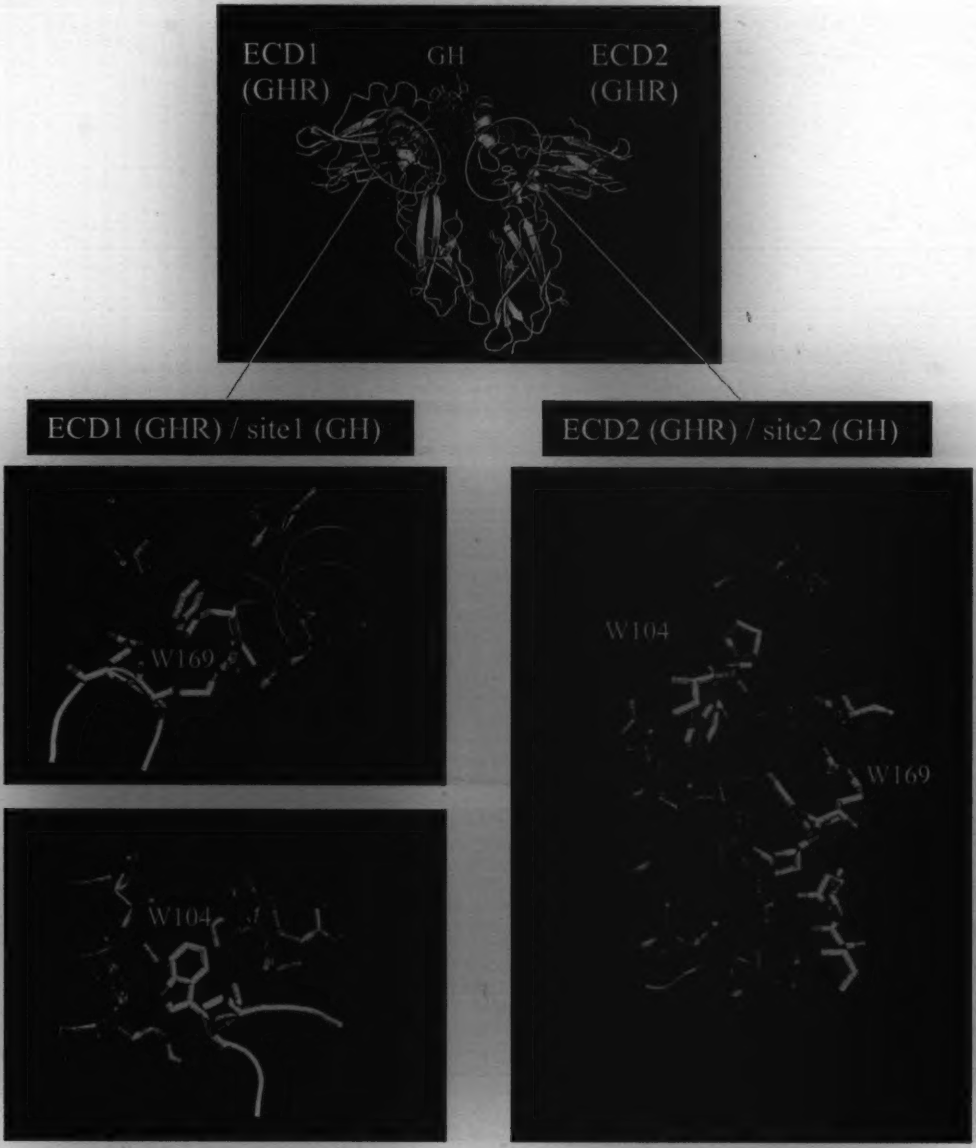
ECD1 (GHR)
GH
ECD2 (GHR)
ECD1 (GHR) / site1 (GH)
ECD2 (GHR) / site2 (GH)
W169
W104
W104
W169

Fig. 1.8. Representation of the two binding sites of GH:2GHR. GH binds two GH receptors (GHR) to form the heterotrimer GH:2GHR. Two residues (tryptophan (W) 104 and 169) in each GHR extracellular domain (ECD, represented in yellow) are crucial for the binding to GH at GH's site 1 and GH site 2 (represented in purple).

receptor facilitating subsequent site 2 binding to the second receptor. Site 2 binding would lower the second receptor and rotate it. This, in turn, would allow the two bound JAK2s to be brought in close proximity and transphosphorylate each other, and the signal transduction to be initiated (Brown et al., 2005). GH induced receptor rotation via sequential GH site 1 and site 2 binding to the GHR dimer would thus be crucial to initiate a cellular response. The GHR mediates the action of GH within target tissues, which may be general (e.g. mitosis, mRNA and protein synthesis) or specific (e.g. serine protease inhibitor 2.1 transcription) (Schwartz et al., 2002) and may reflect endocrine, autocrine / paracrine and possibly intracrine modes of action.

1.2.3 Growth hormone: An *endocrine*, *autocrine / paracrine*, and *intracrine*

GH acts as a classical *endocrine*, i.e. it acts at distant targets after its release into the systemic circulation (Harvey et al., 1995). It also acts as an *autocrine*, i.e. on the cell that synthesized it, as a *paracrine*, i.e. on a cell neighbouring to the one that synthesized it, and maybe as an *intracrine*, i.e. within a cell that synthesized it.

1.2.3.1 Growth hormone: an endocrine hormone

GH is classically considered to be an endocrine with direct actions on target cells or actions mediated by IGF-1. This theory has been refined over the past decades, since the discovery (in 1957) that GH action on bone growth was indirect (Salmon and Daughaday, 1957) and is based upon the "somatomedin"hypothesis.

1.2.3.1.1 Endocrine GH: site of synthesis

Endocrine GH is synonymous of pituitary GH. In mammals, GH is primarily produced by somatotrophs in the adenohypophysis (Daniel et al., 1964). In birds, the anterior pituitary gland is divided into two lobes; somatotrophs are the dominant secretory cell types in the caudal lobe but they can also be found in small proportion in the cephalic lobe (Hull and Harvey, 2002). Endocrine growth hormone is released into the systemic circulation and acts on distant target sites.

1.2.3.1.2 The history of the somatomedin hypothesis

The growth promoting effects of a pituitary "substance" was first established in the 1920s (Evans and Long, 1921, 1922; Evans and Simpson, 1931). This growth promoting ability was later attributed to purified GH (Li et al., 1945) and shown to be mediated by circulating IGF-1. Indeed, injection of GH to hypophysectomized rats promoted the broadening of epiphyseal plates and their sulfate incorporation *in vivo* but not *in vitro* (Murphy et al., 1956; Salmon and Daughaday, 1957). It was hypothesized that a serum-derived "sulfation factor", later identified as somatomedin C (Daughaday et al., 1972), and then as IGF-I (Klapper et al., 1983; Daughaday et al., 1987) mediates the action of pituitary GH on skeletal and soft tissue growth (Salmon and Daughaday, 1957). This initial somatomedin hypothesis was later modified. In the 1980s, a series of experiments strongly suggested that, in addition to circulating IGF-1, locally produced IGF-1 promoted bone growth in an autocrine / paracrine manner (Isaksson et al., 1982; Isaksson et al., 1987). This local IGF-1 appeared to be regulated by serum GH, as bone IGF-1 mRNA increased upon GH injection to cartilage growth plate of hypophysectomized rats (Isgaard et al., 1988). Moreover, GH also had the ability to promote the proliferation of cells in hypophysectomized rat tibial growth plate (Ohlsson et al., 1992). Thus, it was proposed that GH promotes bone growth directly and indirectly via both hepatic and bone-derived IGF-1. A series of experiments conducted in the late 1990s suggested further that circulating IGF-1 did not promote bone growth, since conditional knockout mice displayed normal bone growth in the absence of hepatic IGF-1 and of 75% circulating IGF-1, but induces a negative feedback control to regulate GH secretion (Sjogren et al., 1999; Yakar et al., 1999). One of these groups has, however, nuanced this minimalist role for circulating IGF-1, as liver IGF-1 and acid labile subunit (ALS, a serum IGF-1 binding protein) conditional double knockout (but not ALS knockout mice) do display a strong reduction in body growth associated with a 85-90% decrease in circulating IGF-1 (Yakar et al., 2002). To reconcile these data with the single knockout studies, it was deduced that circulating IGF-1 concentration must be above a threshold to promote bone growth (Yakar et al., 2002) and that the IGF-1: IGF-binding protein (IGF-BP) -3: ALS ternary complex (the major bound form of serum IGF-1) and not IGF-1 alone is important for bone acquisition (Yakar et al., 2006).

1.2.3.1.3 Growth hormone: beyond the somatomedin hypothesis

Whilst the somatomedin hypothesis has greatly added to the understanding of GH and IGF-1 in growth, it has somewhat contributed to the common neglect of other aspects of GH roles, especially its direct functions and its various modes of action. Indeed, GH affects many biological processes beyond bone and tissue growth. GH has been shown to be involved in carbohydrate, lipid and protein metabolism, osmoregulation, reproductive, immune, endocrine and neural functions (Harvey et al., 1995). Moreover, many GH actions are not mediated by IGF-1 but are either direct or mediated by other growth factors. For instance, GH has been shown, independently of IGF-1, to facilitate skeletal muscle cell fusion (Sotiropoulos et al., 2006), to increase the size of cardiomyocytes in neonatal rats (Lu et al., 2001), to promote the expansion of growth plate germinal zone in mice (Jo et al., 1999), to induce the proliferation of pancreatic beta cells (Billestrup and Nielsen, 1991) to promote the expression of BMP-2 and -4 in human dental pulp fibroblasts (Li et al., 1998), and to promote the proliferation of trophectoderm cells in mouse blastocysts (Markham and Kaye, 2003). In addition to IGF-1, GH has been shown to promote the expression of other growth factors: of FGF-2 in rat cartilage *in vivo* and *in vitro* (Izumi et al., 1995), of EGF in rat kidneys (Rogers et al., 1994), of hepatocyte growth factor (HGF) in rat liver (Ekberg et al., 1992), of TGF-β (in combination with insulin and heparin) in human hepatoma cell line (Zvibel et al., 1991), of interleukin-6 (IL-6) in human osteoblasts (Swolin and Ohlsson, 1996) and IL-1 in bovine thymic stromal cells (Tseng et al., 1997), and of BMP-2 and -4 in the rat developing tooth, independently of IGF-1 (Li et al., 1998; Li et al., 2001).

1.2.3.2 Growth hormone: an autocrine / paracrine growth factor

In addition to its role as an endocrine, much evidence strongly suggests that GH acts as an autocrine / paracrine growth factor. Autocrine / paracrine functions are characterized by the presence of GH mRNA and protein within a tissue, by the localization of GHR mRNA and protein within this tissue or in an adjacent tissue and, most importantly, by endogenous actions of GH within this GHR expressing tissue.

1.2.3.2.1 Extrapituitary GH

Many studies have demonstrated the widespread expression of GHR in numerous tissues (Lincoln et al., 1990; Lobie et al., 1990b; Lobie et al., 1990c; Lobie et al., 1990a; Garcia-Aragon et al., 1992) providing the foundations of the pleiotropic roles of endocrine GH. Less attention has been paid to the wide distribution of GH in tissues other than the pituitary; also referred to as "extrapituitary GH". GH has, nevertheless, been shown to be distributed in many adult tissues including those of the nervous system

(Harvey and Hull, 1997). In birds, GH is detected in hypothalamic and extrahypothalamic tissues in the chicken brain (Render et al., 1995), and in turkey and ring dove brain (Ramesh et al., 2000). GH-like immunoreactivity is also found in the hypothalamus and median eminence, and extrahypothalamic tissues of the rat brain (Lechan et al., 1981; Lechan et al., 1983) and in the hypothalamus of fish (Hansen and Hansen, 1982). In addition, GH has also been detected in the immune system in the spleen, thymus, bone marrow, peripheral blood leukocytes, and ileum Peyer's patches in rodents (Weigent et al., 1988; Weigent and Blalock, 1989; Kooijman et al., 1997a; Recher et al., 2001), and in humans (Wu et al., 1996; Kooijman et al., 1997b; de Mello-Coelho et al., 1998; Wu et al., 1999) and in the chicken immune system, in the thymus, spleen and bursa of Fabricius (Luna et al., 2005). GH has also been detected in the mammary gland (Mukhina et al., 2006), in the fetal reproductive tract (Nguyen et al., 1996) and in blastocysts (Pantaleon et al., 1997) in mice.

The presence of GH protein might reflect the mere sequestration of pituitary GH. However, GH mRNA is also found in many tissues where GH protein is detected, indicating that GH is derived from local synthesis. In the nervous system, the *GH* gene is transcribed in hypothalamic and extrahypothalamic tissues in the chicken brain (Render et al., 1995), in the fish brain (Yang et al., 1999) and in many neural tissues of the rat CNS, such as the cortex, caudate putamen, thalamus, hypothalamus and hippocampus (Gossard et al., 1987; Martinoli et al., 1991). In addition, GH mRNA is translated within immune tissues, as GH has been shown to be synthesized *de novo* by rat leukocytes (Weigent et al., 1988) and human thymocytes (Sabharwal and Varma, 1996). GH mRNA has also been detected within mammary tissues in mouse (Mukhina et al., 2006) and in human (Mol et al., 1995; Raccurt et al., 2002). GH mRNA is found in malignant mammary tumors in human (Mol et al., 1995; Raccurt et al., 2002), where GH proteins levels are elevated (Raccurt et al., 2002). GH mRNA has been detected in the fetal mouse reproductive tract (Nguyen et al., 1996) and in the morula in preimplantation embryos (Pantaleon et al., 1997).

The precise function of GH in extrapituitary sites of production is uncertain, especially in tissues where both pituitary and extrapituitary GH could act. It has, however, been shown that extrapituitary GH has roles in its own right that are independent and / or different from endocrine GH (Xu et al., 2005; Zhu et al., 2005).

1.2.3.2.2 *Bona fide* autocrine / paracrine GH action

In many tissues, GH protein, mRNA and GHRs are colocalized. This strongly suggests autocrine / paracrine mode of actions for GH. For instance GH, like GHR, is present in hypothalamic and extrahypothalamic tissues. GHR mRNA is present in the rat hypothalamus (Minami et al., 1993) and GH binding sites are found in the hypothalamus of chicken (Attardo and Harvey, 1990), rats (Mustafa et al., 1994) and teleost fish (Perez Sanchez et al., 1991). GHR / BP mRNA and protein and GH binding are also detected in extrahypothalamic tissues in rats, rabbits, human, chicken and fish (Fraser et al., 1990; Perez Sanchez et al., 1991; Mustafa et al., 1994; Castro et al., 2000; Harvey and Hull, 2003). The distribution of GH and GHR mRNA and protein in the developing nervous system is described in detail (see below). Within the immune system, the GHR gene has also been shown to be synthesized within rodent bone marrow, spleen and thymus (Mertani and Morel, 1995; Sumita et al., 2005) and within human thymocytes (de Mello-Coelho et al., 1998). GHR protein, in turn, is detected in human peripheral lymphocytes, especially in the surface of B cells (Badolato et al., 1994; Rapaport et al., 1995; Bresson et al., 1999). GHR mRNA and protein are also present in healthy mammary tissues (Lincoln et al., 1990; Ilkbahar et al., 1999) and in mammary tumors in humans (Mertani et al., 1998). GHR mRNA and protein have also been localized in preimplantation embryos from the two-cell stage to the blastocyst stage in mouse (Ohlsson et al., 1993; Terada et al., 1996; Pantaleon et al., 1997) and GHR mRNA was detected earlier in the fertilized egg (Pantaleon et al., 1997).

Autocrine / paracrine modes of action can be demonstrated exclusively by determining endogenous roles for GH, by altering its local expression at sites of GH action. For instance in the developing neural retina, the immunoneutralization of endogenous GH *in vivo* induces a 3.5-5.5 fold increase in the number of cell death, depending on the antibody used, including the number of apoptotic RGCs *in vitro*, indicating that GH promotes ganglion cell survival (Sanders et al., 2005). Similarly, in mice the immunoneutralization of endogenous GH induces an increase in the number of neurons in cells extracted from the subventricular zone, suggesting that GH prevents the differentiation of neurons (Turnley et al., 2002). In the immune system, the immunoneutralization- or antisense oligonucleotide-inhibition of endogenous GH prevents the proliferation of human thymic epithelial cells (Sabharwal and Varma, 1996), and of human and rat lymphocytes (Weigent et al., 1991; Lattuada et al., 1996). Immunoneutralization of GH also inhibits the GH-induced ^{3}H-thymidine uptake by human thymic epithelial cell cultures (Sabharwal and Varma, 1996). Autocrine GH, but not endocrine GH, is also involved in oncogenesis of mammary cells. Indeed, the overexpression of the *GH* gene transfected to human and mouse mammary cell lines stimulates proliferation and survival of normal and carcinoma mammary epithelial cell lines (Xu et al., 2005; Zhu et al., 2005; Mukhina et al., 2006). This autocrine GH also induces mammary carcinoma cell migration and invasion *in vitro* and *in vivo* (Mukhina et al., 2004). Interestingly, exogenous GH cannot generate tumor formation nor invasion of mammary cells, contrary to autocrine GH (Xu et al., 2005; Zhu et al., 2005). In fact, exogenous and autocrine GH differentially induce the transcription of about 300 different genes in mammary carcinoma cells (Xu et al., 2005). In mouse preimplantation embryos, the immunoneutralization of endogenous GH inhibits the proliferation of trophectoderm cells in blastocysts (Markham and Kaye, 2003). In the male reproductive tract, the immunoneutralization of endogenous GH blocks the differentiation of the fetal Wolffian duct in the mouse *in vitro* (Nguyen et al., 1996). Finally in carp pituitaries, the immunoneutralization of GH inhibits basal and growth hormone releasing factors-induced GH gene expression and GH release (Zhou et al., 2004b; Zhou et al., 2004a), suggesting GH exerts an ultra short feedback regulation on its own secretion.

1.2.3.3 Growth hormone: an intracrine messenger

The localization of GH and GHR in the nucleus suggests that GH might also act as an intracrine within target tissues.

1.2.3.3.1 Nuclear Growth hormone

GH has been detected in the cell nucleus (Morel et al., 1993; Pantaleon et al., 1997; Harvey et al., 2001). GH could thus act as a "strict" intracrine (i.e. within the same cell when cytosolic GH translation is followed by nuclear translocation), or "broad" intracrine (i.e. GH is first secreted then endocytosed into the cytoplasm and subsequently translocated to the nucleus). Although there is no evidence, to date, of a strict intracrine action for GH, exogenous GH has been shown to translocate to the nucleus via a retrograde transport through the endosomal pathway system (Lobie et al., 1994b; Mertani et al., 1996). The translocation mechanisms of GH from the cytosol or nuclear membrane to the nucleus are presently unknown. In particular, the mechanisms leading GH translocation to the nucleus via nuclear pores remain to be elucidated, especially as GH does not have an identified nuclear localization signal (NLS) (Miller and Eberhardt, 1983). GH could thus associate with a NLS containing chaperone protein or passively diffuse through nuclear pores, as it is less than 50K (Wente, 2000; Macara, 2001).

1.2.3.3.2 Nuclear GHR/GHBP

GHR and GHBP have been detected within both outer and inner nuclear membranes, chromatin and nucleoplasm of chicken, rabbit, rat, mouse, and human (Lobie et al., 1990b; Lobie et al., 1991; Fraser and Harvey, 1992; Garcia-Aragon et al., 1992; Lobie et al., 1992; Lobie et al., 1994a; Pantaleon et al., 1997; Lincoln et al., 1998; Harvey et al., 2000). However, GHR does not have a known NLS either (Lobie et al., 1994a). In Chinese hamster ovary (CHO) cells transfected with GHR, GHR / BP appears to have a defined subnuclear location within the euchromatin and the nuclear membranes (Mertani et al., 1994) and GHR has been reported to be anchored to the nucleus via part of its intracellular domain (amino acid 294-454) (Lobie et al., 1994). GH translocation occurs in a receptor dependant manner *in vivo* and *in vitro,* since nuclear translocation of GH requires this intracellular domain of the GHR (Lobie et al., 1994b) and the dimerization of the receptor (Mertani et al., 2003). Interestingly, JAK2 activation and receptor phosphorylation is not required for nuclear translocation, but might be involved in preventing GH accumulation in the nucleus (Mertani et al., 2003).

In summary, although GH is classically considered to be a strict endocrine, it is also an established autocrine / paracrine and possibly an intracrine. GH might similarly act as a hormone and / or as a growth factor in the developing nervous system.

1.2.4 Growth hormone: roles in the nervous system development

The relatively recent discovery that GH and GHR are widely distributed in developing neural tissues suggests that GH is intimately involved in neural development, although very little is known of these roles[5].

1.2.4.1 Sites of GH production during nervous system development

1.2.4.1.1 Placental and pituitary GH

During pregnancy, circulating levels of maternal GH are elevated in most mammals (Kelly et al., 1976), and in human, the placenta (and not the pituitary) is the major source of GH from the second half of pregnancy (Frankenne et al., 1988; Mirlesse et al., 1993).

In large vertebrates, pituitary GH appears early in development. For instance, GH-secreting cells are detected in the fetus as early as the second month of the 9 month gestation period in bovines (Dubois, 1971) and humans (Asa et al., 1986). In small vertebrates, GH is detected in the pituitary and plasma in the last third of gestation. For instance in rodents (in rats), GH is first detected within pituitary somatotrophs at E18 of the 22 day gestation period within pituitary somatotrophs (Nogami et al., 1989; Garcia-Aragon et al., 1992) and by E19 in the circulation (Strosser and Mialhe, 1975). Rat serum GH concentrations are high during the perinatal period but decrease shortly after birth (Strosser and Mialhe, 1975). In chicken, GH immunoreactivity is detected within the pituitary gland by E12 of the 21 day incubation period within the pituitary gland (Malamed et al., 1993), but it is not released from somatotrophs before E16 (Porter et al., 1995) and it is not found within the plasma before E17 (Harvey et al., 1979). Thus, a significant period of vertebrate gestation / incubation (and embryonic and fetal growth) occurs in the absence of pituitary / endocrine GH.

However, as nervous system development continues after birth, affecting glial cell maturation and neuronal connections particularly, pituitary GH might influence the development of nervous tissues perinatally and neonatally.

1.2.4.1.2 Neural GH

GH immunoreactivity (Table 1.2) is present in numerous tissues within the developing nervous system. The distribution of GH is best known in the early chick

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[5] The distribution and roles of GH in the neural retina will be reviewed in a separate section.

Table 1.2. Prenatal and neonatal GH protein distribution within the nervous system (except for the neural retina)

Species	Tissue	Day	Reference
Chicken	Neural tube	E3-7	(Harvey et al., 2000)
Chicken	Telencephalon	E3	(Harvey et al., 2001)
Chicken	Diencephalon	E3, E7	(Harvey et al., 2001) (Murphy and Harvey, 2001)
Chicken	Mesencephalon	E3, E7	(Harvey et al., 2001) (Murphy and Harvey, 2001)
Chicken	Metencephalon	E3	(Harvey et al., 2001)
Chicken	Myelencephalon	E3	(Harvey et al., 2001)
Chicken	Brain	E21	(Harvey et al., 2003)
Chicken	Cerebral cortex (cells and fibers)	E14	(Harvey et al., 2001)
Chicken	Olfactory pit	E3	(Harvey et al., 2001)
Chicken	Otic vesicles	E3	(Harvey et al., 2001)
Chicken	Choroid plexus	E14	(Harvey et al., 2001)
Chicken	Ependymal cells	E7 E19	(Harvey et al., 2001) (Murphy and Harvey, 2001)
Chicken	Cerebellum	E19	(Harvey et al., 2001)
Chicken	Spinal cord (ventral and dorsal horn; outer marginal layer and basal plate mantle)	E7	(Murphy and Harvey, 2001)
Chicken	Ventral root and dorsal root ganglia	E7	(Murphy and Harvey, 2001)
Chicken	Vth (semilunar) nerve ganglia	E3, E7	(Harvey et al., 2001)
Chicken	VIIth (facial) nerve ganglion	E3, E7	(Harvey et al., 2001)
Chicken	VIIIth (acoustic) nerve ganglion	E3, E7	(Harvey et al., 2001)
Chicken	IXth (glossopharyngeal) nerve ganglion	E3	(Harvey et al., 2001)
Chicken	Accessory ganglion	E7	(Murphy and Harvey, 2001)
Chicken	Trigeminal nerve	E7	(Murphy and Harvey, 2001)
Chicken	Vagal nerve	E7	(Murphy and Harvey, 2001)
Mouse	Ventricular zone (cortex)	E14	(Turnley et al., 2002)
Rat	Whole brain	E10	(Hojvat et al., 1982b)
Rat	Cortex	E16-P0*	(Hojvat et al., 1982b)
Rat	Thalamus	E16-P0*	(Hojvat et al., 1982b)
Rat	Hypothalamus	E16-P0*	(Hojvat et al., 1982b)
Rat	Amygdala	E16-P0*	(Hojvat et al., 1982b)
Rat	Hippocampus	E16-P0*	(Hojvat et al., 1982b)
Human	Brain (low)	Wk8-32	(Costa et al., 1993)

* peaks at E21; P: Postnatal day; Wk: week

embryo. Indeed, GH has been found early on in the neural tube, in the five brain vesicles and in the developing spinal cord in the CNS (Harvey et al., 2000, 2001; Murphy and Harvey, 2001) long before its appearance in the pituitary gland. GH is also found in many nerves and ganglia, such as the trigeminal and vagal nerves, in the dorsal root ganglion, the ventral root and in the Vth, VIIth, VIIIth and IXth nerve ganglia (Harvey et al., 2001; Murphy and Harvey, 2001). Later in development, GH expression becomes more restricted to specific cell types and tissues, and it is found in the brain in cerebral cortex, cerebellum and ependymal cells (Harvey et al., 2001; Harvey et al., 2003). Rodents are altricial (naked and blind at birth) species and as such their nervous sytem is not fully developed at birth. GH has been found in perinatal rat brain, in the cortex, thalamus, hypothalamus, hippocampus, and in the amygdala (Hojvat et al., 1982a). GH is also found in the fetal rat brain and mouse cerebral cortex in the ventricular zone (Hojvat et al., 1982a; Turnley et al., 2002) and in the fetal human brain (Costa et al., 1993) before GH is detected within the developing pituitary. In fetal rats, brain GH levels rise dramatically few days before birth and drop within 24 hours after birth (Hojvat et al., 1982b).

1.2.4.2 Sites of GH action during nervous system development

GHR / BP mRNA (Table 1.3) and protein distribution is also widespread in the developing nervous system. Indeed, GHR mRNA has been detected in fetal and newborn mouse primary neurospheres and in the progeny of adult stem cells (Turnley et al., 2002). In rats, GHR mRNA is found within the hypothalamus (Hasegawa et al., 1993), in dorsal root ganglia, cranial nerve ganglia, and in the internal granular layer of the olfactory bulb, where no IGF-1 mRNA is found, suggesting IGF-1 independent roles for GH (Edmondson et al., 1995). In the rat hypothalamus, GHR mRNA increases between E15 and postnatal day (P) 7 and remains elevated until P35, and the ratio between GHR and GHBP transcripts also increases with its lowest levels (less than 2) occurring during the fetal period (Hasegawa et al., 1993). In the postnatal rat brain, GHR mRNA is elevated shortly after birth (P2) and drops by more than two fold by P10 (Lobie et al., 1993). In the postnatal rabbits, GHR / BP mRNA is detected in the layers 2 and 3 of the cerebral cortex, in the hippoccampus within granule and pyramidal cells, in the lateral basal hypothalamus, in the dorsal thalamus, in circumventricular organs, and in Bergmann

Table 1.3. Prenatal and neonatal GHR / BP mRNA distribution in the developing nervous system (except for the neural retina)

Species	Tissue	Day	Reference
Mouse	CNS (GHBP)	E12-P18	(Turnley et al., 2002)
Mouse	Primary neurospheres (GHR)	E13, P0	(Turnley et al., 2002)
Rat	Inner olfactory bulb	E16.5-20.5	(Edmondson et al., 1995)
Rat	Hypothalamus	E15-P35	(Hasegawa et al., 1993)
Rat	Dorsal root ganglia Cranial nerve ganglia	E16.5-20.5	(Edmondson et al., 1995)
Rabbit	Cerebral cortex	P20	(Lobie et al., 1993)
Rabbit	Thalamus	P20	(Lobie et al., 1993)
Rabbit	Hypothalamus	P20	(Lobie et al., 1993)
Rabbit	Hippocampus	P20	(Lobie et al., 1993)
Rabbit	Cerebellum	P20	(Lobie et al., 1993)
Rabbit	Circumventricular organs	P20	(Lobie et al., 1993)
Bovine	Spinal cord	E40	(Kolle et al., 1998)
Bovine	Splanchnic nerves	E40	(Kolle et al., 1998)
Bovine	Spinal ganglia	E40	(Kolle et al., 1998)
Human	CNS	Wk 14.8	(Zogopoulos et al., 1996)

glial cells of the cerebellum (Lobie et al., 1993). Finally in human, GHR mRNA is detected in the CNS in the third month of gestation (Zogopoulos et al., 1996).

GHR / BP immunoreactivity (Table 1.4) is detected in the early chick embryo in the neural tube (Harvey et al., 2000), in the five brain vesicles, the otic vesicles and in the Vth, VIIth and IXth nerve ganglia (Harvey et al., 2001). In embryonic mouse, GHR / BP immunoreactivity is detected in the cerebral cortex, migrating away form the ventricular zone (Turnley et al., 2002). In the fetal rat, GHR / BP immunoreactivity is also detected in neurons of the five brain vesicles and spinal cord (Garcia-Aragon et al., 1992). In addition, a strong signal is detected in schwann cells and in neurons of the dorsal root and nerve ganglia including the trigeminal ganglion (Garcia-Aragon et al., 1992). The cells that display the most intense immunoreactivity are ganglion cells and ependymal cells, especially in the choroid plexus, although other neurons and macroglial cells are also immunoreactive (Garcia-Aragon et al., 1992). The GHR / BP immunoreactivity of fiber tracts are only moderate (Garcia-Aragon et al., 1992). GHR / BP immunoreactivity increases from E12 to E18 but mostly between E16 and E18 (Garcia-Aragon et al., 1992). GHR / BP immunoreactivity is also detected in developing rat nervous tissues after birth and it is found to be ubiquitously distributed within all regions and cell types of the CNS, albeit with differences in *in situ* hybridization labeling intensity (Lobie et al., 1993). Thus the immunoreactive signal associated with GHR / BP is mostly intense in the multiform cell layer of frontal lobe, neurons of the thalamus, Purkinje cells of the cerebellum, in the nucleus corporis trapezodei and neurons of the brain stem, in astrocytes of the cingulum and in ependymal cells of the choroid plexus, ventricular lining and pia matter (Lobie et al., 1993). In the postnatal rabbit CNS, the highest immunoreactive intensity is observed in the cerebral cortex, in the hippocampus, in the inferior and superior colliculi, in the thalamus, hypothalamus, in the circumventricular organs and in the brain stem (Lobie et al., 1993). GHR / BP immunoreactivity decreases during postnatal development in rat and rabbit CNS (Lobie et al., 1993). In the bovine embryo, GHR immunoreactivity is particularly strong in the splanchnic nerves as well as in the spinal cord within motoneurons and in spinal ganglia, and colocalize with GHR mRNA in these tissues (Kolle et al., 1998). Finally in human, GHR / BP is detected in the somata of cerebral cortex neurons in the end of the third trimester (Hill et al., 1992).

Table 1.4. Prenatal and neonatal GHR distribution in the developing nervous system (except for the neural retina)

species	Tissue	Day	reference
Chicken	Neural tube	E2-4	(Harvey et al., 2000)
Chicken	Telencephalon	E3	(Harvey et al., 2001)
Chicken	Diencephalon	E3	(Harvey et al., 2001)
Chicken	Mesencephalon	E3	(Harvey et al., 2001)
Chicken	Mylencephalon	E3	(Harvey et al., 2001)
Chicken	Metencephalon	E3	(Harvey et al., 2001)
Chicken	Otic vesicles	E3	(Harvey et al., 2001)
Chicken	Vth (semilunar) cranial ganglia	E3	(Harvey et al., 2001)
Chicken	VIIth (Facial/acoustic) ganglion	E3	(Harvey et al., 2001)
Chicken	IX (Glossopharyngeal) ganglion	E3	(Harvey et al., 2001)
Mouse	Cerebral cortex	E14	(Turnley et al., 2002)
Rat	CNS neurons	E18	(Garcia-Aragon et al., 1992)
Rat	Telencephalon	E18	(Garcia-Aragon et al., 1992)
Rat	Diencephalon	E18	(Garcia-Aragon et al., 1992)
Rat	Mesencephalon	E18	(Garcia-Aragon et al., 1992)
Rat	Mylencephalon	E18	(Garcia-Aragon et al., 1992)
Rat	Metencephalon	E18	(Garcia-Aragon et al., 1992)
Rat	Choroid plexus Ventricular lining	E16-18, P10	(Garcia-Aragon et al., 1992) (Lobie et al., 1993)
Rat	Spinal cord	E18	(Garcia-Aragon et al., 1992)
Rat	Dorsal root	E12-18	(Garcia-Aragon et al., 1992)
Rat	cranial ganglia (including trigeminal ganglion)	E12-18	(Garcia-Aragon et al., 1992)
Rat	Schwan cells (ANS)	E12-E18	(Garcia-Aragon et al., 1992)
Rat	Ventricular zone (periphery)	E14	(Turnley et al., 2002)
Rat	Frontal lobe	P10	(Lobie et al., 1993)
Rat	Olfactory bulb	P10	(Lobie et al., 1993)
Rat	Hippocampus	P10	(Lobie et al., 1993)
Rat	Dendate Gyrus	P10	(Lobie et al., 1993)
Rat	Amygdala	P10	(Lobie et al., 1993)
Rat	Thalamus	P10	(Lobie et al., 1993)
Rat	Hypothalamus	P10	(Lobie et al., 1993)
Rat	Superior and inferior colliculus	P10	(Lobie et al., 1993)
Rat	Cerebellum	P2-28	(Lobie et al., 1993) (Lincoln et al., 1994)
Rat	Brain stem	P10	(Lobie et al., 1993)
Rat	Glial cells	P10	(Lobie et al., 1993)
Rabbit	Cerebral cortex	P10-20	(Lobie et al., 1993)
Rabbit	hippocampus	P10-20	(Lobie et al., 1993)
Rabbit	Inferior and superior colliculus	P10-20	(Lobie et al., 1993)

Rabbit	Thalamus	P10-20	(Lobie et al., 1993)
Rabbit	Hypothalamus	P10-20	(Lobie et al., 1993)
Rabbit	Cerebellum	P10-20	(Lincoln et al., 1994)
Rabbit	Brain stem	P10-20	(Lobie et al., 1993)
Rabbit	circumventricular organs	P10-20	(Lobie et al., 1993)
Bovine	Spinal cord (motoneurons of the ventral horn)	E40	(Kolle et al., 1998)
Bovine	Spinal ganglia	E40	(Kolle et al., 1998)
Bovine	Splanchnic nerves	E40	(Kolle et al., 1998)
Human	Cerebral cortex	Wk14-16	(Hill et al., 1992)

Some studies (Garcia-Aragon et al., 1992; Hill et al., 1992; Lobie et al., 1993; Kolle et al., 1998; Harvey et al., 2000, 2001) did not detect GHR *per se* but the extracellular domain of GHR or its binding protein (GHBP). Thus, the GHR / BP expressing tissues might not be *bone fide* GH target sites, although this is unlikely, as GH has been shown to alter the development of the nervous system.

1.2.4.3 GH action during nervous system development

1.2.4.3.1 Phenotype of the nervous system in GH deficiency and excess

An impaired nervous system development has been observed in GHR or GH deficient organisms. In humans, patients suffering from Laron dwarfism[6] display slow motor development and slight mental retardation in some cases (Laron et al., 1968; Godowski et al., 1989) and patients suffering from Jacobsen syndrome, characterized by GH deficiency, present CNS abnormalities (Pivnick et al., 1996). Similarly in rodents, retarded CNS growth, including a poor development of the pineal gland and of suprachiasmatic nucleus of hypothalamus, is observed in Snell dwarf mice[7] (dw/dw), and in Little mouse[8], and is associated with poor locomotor activity and impaired periodicity (Noguchi et al., 1983; Noguchi et al., 1986; Camper et al., 1990; Godfrey et al., 1993; Noguchi, 1996). Indeed in Snell dwarf mice, the motor cortex neurons have smaller perikarya and display an impaired dendritic growth compared to control (Noguchi et al., 1983). Axon development might be impaired, since a reduction in fiber number is observed in the corpus callosum (Noguchi et al., 1983). Synaptogenesis might also be decreased, based on the observations that Thy-1 is especially associated with synapses and that Thy-1 antigens are reduced in cerebrum, cerebellum and brain stem in these animals (Noguchi et al., 1983; Noguchi, 1996). In addition, the brain of these mice might be hypomyelinated, as a reduction in fibers positive for myelin basic protein is found in the corpus callosum and a reduction in CNPase (2',3'-cyclic nucleotide 3'-phophohydrolase) activity, a marker of myelin formation, is present in cerebrum and brain stem homogenates (Noguchi et al., 1983; Noguchi, 1996). This reduction in myelination might be associated with a decrease in glial cell proliferation (Noguchi, 1996), since these animals display suppressed thymidine incorporation and lower cerebral DNA content. CNPase activity and growth of dendrites and neuronal perikarya are restored to normal upon administration of exogenous bovine GH (bGH) within 20 days after birth, in association with thyroxine (Noguchi et al., 1982, 1984), but not later (Noguchi et al., 1984; Sugisaki et al., 1985; van Buul-Offers and Van den Brande, 1985), suggesting there is a restricted neonatal window for GH neural action. Like the Snell dwarf mice, the dendrites of neurons of the motor cortex are also poorly developed in Little mice. Cerebrum and cerebellum have a reduced weight, DNA and RNA content, and the cerebrum has a reduced CNPase activity, suggesting that the brain of these mice might be hypomyelinated (Noguchi et al., 1985; Noguchi and Sugisaki, 1985; Noguchi, 1996). Again, administration of bGH alone during the first 20 days postnatally, but not later, induces an increase in cerebrum weight, DNA and RNA content, and CNPase activity (Noguchi et al., 1988; Noguchi, 1996). This bGH treatment is hypothesized to improve myelination by increasing glial cell proliferation (Noguchi et al., 1988). As bGH alone can improve cerebral development in Little mice, it is thought that GH can promote brain development independently, at least in part, in addition to its complementary or synergistic action with thyroxine (Noguchi et al., 1988). The results are, however, disputed by Lehman et al., who found that total myelin content in Little mouse is not reduced when normalized to brain size; hypomyelination would be a direct result of decreases neuronal proliferation (Lehman et al., 1999). However, recent evidence also indicates that GH induces glial cell proliferation in the cortex of fetal rats (Ajo et al., 2003) and promotes myelination and oligodendrocyte development in rat CNS (Palacios et al., 2001). In addition, brain and spinal cord weight is increased in transgenic mice overexpressing a methallothionein-I promoter / GH fusion gene, as well as the size of lumbar spinal motoneuron nucleus and cell body (Chen et al., 1997). This effect is unlikely to be mediated by IGF-1, as lumbar motoneuron are increased in size in transgenic mice overexpressing GH but not in mice overexpressing IGF-1 (Chen et al., 1997). Taken together, these data strongly suggest that GH is involved in the development of the nervous system. Indeed, GH has recently been shown to be directly associated with cell proliferation, cell differentiation and cell death.

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[6] Laron dwarfism is an autosomal recessive disorder characterized by abnormal GHR (Godowski et al., 1989).

[7] Snell dwarf mice (dw/dw) are characterized by a deficit in GH, TSH and PRL due to pit-1 mutation (Camper et al., 1990).

[8] Little mouse are characterized by absent circulating GH due to a mutation in the GHRH receptor gene (Godfrey et al., 1993; Noguchi, 1996).

1.2.4.3.2 GH promotes neural proliferation

GH promotes cell proliferation in the fetal rat brain (Sara et al., 1974; Ajo et al., 2003). In rats, addition of exogenous human GH (hGH) to cell culture medium promotes the proliferation of cells, indicated by an increase in BrdU (bromodeoxyuridine) incorporation in cerebral cortical precursor cells (Ajo et al., 2003). Interestingly, GH induces an increase in the number of nestin / BrdU positive cells (nestin is a marker of precursor cells) in 3-day cultures whilst this number decreases in 7-day cultures compared to controls, suggesting GH might also stimulate differentiation (Ajo et al., 2003). More specifically, GH promotes the proliferation of cells of neural and astrocytic lineage extracted from the E14 and E17 fetal rat cerebral cortex, respectively (Ajo et al., 2003). Indeed, GH treatment induces an increase in the number of β-tubulin (a marker for neurons) / BrdU positive cells and of GFAP (glial fibrillary acidic protein, a marker for glial cells) / BrdU positive cells; (Ajo et al., 2003). This action is mediated by IGF-1, as GH-induced proliferation of fetal rat cortical cells is blocked by IGF-1 antiserum, and as GH stimulates the expression of IGF-1 and IGF-1 receptor β-subunit, and the phosphorylation of IGF-1 receptor β-subunit in E17 cells (Ajo et al., 2003). This might explain the hypomyelination observed in Snell and Little mice (Noguchi et al., 1983; Morisawa et al., 1989).

1.2.4.3.3 GH promotes neural cell differentiation

GH also promotes cell differentiation in the developing nervous system. hGH has been shown to induce the differentiation of E17 fetal rat cerebrocortical neurons in culture, as it induces an increase in the number of cells expressing β-tubulin, while the number of β-tubulin / BrdU expressing cells remains unchanged, suggesting these cells are indeed postmitotic (Ajo et al., 2003). This increase in cell number might also be attributed to cell survival as opposed to differentiation (Ajo et al., 2003). Nevertheless, GH treatment of E17 cortical cells in culture also induces a change in cell morphology, as it promotes the development of mature neuron-type traits with an increase in the number and length of the cellular processes and an overall increase in β-tubulin levels in cultured cells (Ajo et al., 2003). GH might also promote astrocyte differentiation, since the increase in GFAP / BrdU positive cells in E17 rat cortical cell culture is accompanied by morphological changes, including denser and longer cell processes (Ajo et al., 2003).

This action is mediated by IGF-1, as GH-induced differentiation of fetal rat cortical cells is blocked by IGF-1 antiserum (Ajo et al., 2003). Moreover in mice, GH has been shown to promote the differentiation of astrocytes while preventing the differentiation of cortical neurons in stem cell culture (Turnley et al., 2002). GH treatment induces a decrease in the number of βIII-tubulin (a neuronal marker) positive cells but does not alter the number of cells in neurosphere culture derived from neonate mice subventricular stem cell at P0 and in E12.5 neuroepithelial cell culture (Turnley et al., 2002). Conversely, immunoneutralization of GH induces an increase in the number of neurons, indicating endogenous GH inhibits neural progenitor cell differentiation into neurons (Turnley et al., 2002). Recombinant rat GH prevents neuronal (and promote astrocyte) differentiation by downregulating neurogenin-1 (Turnley et al., 2002; Turnley, 2005), a bHLH transcription factor promoting neurogenesis and inhibiting gliogenesis (Sun et al., 2001). GH-mediated downregulation of neurogenin levels and subsequent blockade of neuron differentiation is inhibited by SOCS2, a negative regulator of GH signaling (Turnley et al., 2002). GH promotion of astrocyte differentiation via inhibition of neurogenin-1 is consistent with the hypomyelination detected in Snell dwarf and Little mice (Noguchi et al., 1983; Morisawa et al., 1989) and with the observed decrease in neuron to glial cell ratio in the GHR knockout cortex (Ransome et al., 2004). Thus GH promotes the differentiation of cortical neurons and of glial cells from the cerebral cortex and subventricular zone.

1.2.4.3.4 GH prevents neural cell death

GH is neuroprotective in the neonatal rat brain after hypoxic-ischemic injury (Gustafson et al., 1999; Shin et al., 2004). Subcutaneous injection of GH prevents severe damage to the cerebral hemispheres, including cortical atrophy and the appearance of TUNEL positive apoptotic cells, compared to saline control (Shin et al., 2004). GH neuroprotection is likely to be mediated by the Bcl-2 family of proapoptotic molecules Bax and Bad, as their immunoreactive levels, but not those of Bcl-2, are downregulated in GH treated rats within cerebral cortices (Shin et al., 2004). GH is also suspected to prevent cell death by inhibiting iNOS (inducible nitric oxide synthase), as it decreased the levels of this neurotoxic molecules (Shin et al., 2004).

In summary, GH has been shown to affect proliferation, differentiation and cell survival of developing neural tissues. The possibility that GH is involved in other aspects of the nervous system development, namely cell migration, neuritogenesis, axon guidance, wiring, and vascularization has yet to be directly assessed.

1.2.4.4 Endocrine versus autocrine / paracrine GH in the nervous system development

1.2.4.4.1 GH: a maternal and fetal endocrine?

GH might act as an endocrine during the development of the nervous system. Endocrine GH might be derived from a maternal source or directly from the pituitary of the fetus. Thus endogenous or exogenous GH injected into the circulation or subcutaneously might alter neural development either indirectly (by altering the metabolism, for instance) or directly, assuming that circulating GH can cross the blood-brain barrier (BBB). The possibility that GH crosses the BBB is still somewhat controversial. Early studies have shown that the passage of exogenous GH across the BBB is either absent or limited in monkeys and rodents, including fetal and neonatal rats (Mayberry et al., 1971; Belchetz et al., 1982; Hojvat et al., 1982a). However, recent evidence suggests that exogenous GH does cross the BBB, as peripheral exogenous ^{125}I-rat GH (rGH) is intaken by brain in rats and mice (Pan et al., 2005) and as a correlation exists between cerebrospinal fluid GH and serum GH levels (Harvey and Hull, 2003). It is thus possible that endocrine GH acts directly on GHR in the nervous system.

Maternal endocrine GH has been shown to promote brain development. Subcutaneous injection of bGH into pregnant rats induced an increase in brain to body weight ratio, in cerebral cortex thickness and cell number (Zamenhof, 1942), in total DNA per brain in neonatal pups (Zamenhof et al., 1966), and a hypertrophy of cortical cells and dendritic fields in P33-60 rats (Clendinnen and Eayrs, 1961). As GH does not cross the placenta (Fholenhag et al., 1994), maternal GH likely exerts its effects by increasing nutrient availability to the fetus. Indeed, pregnant rats on a low protein diet have been reported to give birth to pups with low brain weight (Zamenhof et al., 1966).

Fetal and neonatal pituitary GH might promote brain development also. Intraperitoneal GH injections to tadpoles induced an increase in both neurons and glial cells in the cerebral hemispheres (Zamenhof, 1941) and an increase in brain weight and in brain DNA concentration (Hunt and Jacobson, 1970, 1971). GH deficient neonate mice display CNS abnormalities, that are reversed by bGH administration, at least in part

(Noguchi et al., 1983; Noguchi et al., 1986; Noguchi, 1996). In addition, subcutaneous injection of GH in neonatal rats has been shown to be neuroprotective in hypoxic-ischemic brain injury (Gustafson et al., 1999; Shin et al., 2004). It is likely that GH promotes cell survival directly, as BBB permeability to exogenous GH is increased after CNS injury (Mustafa et al., 1995). Thus GH promotes nervous system development as an endocrine.

1.2.4.4.2 GH: an autocrine / paracrine

As GH and GHR are colocalized within the developing PNS and CNS, GH is highly likely to promote the development of the nervous system via autocrine / paracrine mechanisms. In the chick embryo, both GH and GHR / BP immunoreactivity are found in the neural tube, the developing brain vesicles, in the otic vesicles and in the Vth, VIIth and IXth cranial nerve ganglia before pituitary differentiation (Harvey et al., 2000, 2001). In mice, GH and GHR / BP are both present in the cerebral cortex a few days before GH is detected in the differentiating pituitary (Turnley et al., 2002). Within the cortex, GH is located in the ventricular zone while GHR is found to be migrating away from it (Turnley et al., 2002), suggesting paracrine roles for GH. In rats, GHR / BP has been detected in the telencephalon and diencephalon, including the hypothalamus (Garcia-Aragon et al., 1992; Hasegawa et al., 1993) while GH has been detected in telencephalon (in cortex, amygdala, and hippocampus), and in diencephalon (in thalamus and hypothalamus) (Hojvat et al., 1982b) shortly before and after GH is found in the circulation. Finally, GH and GHR are both detected in the human brain, although after GH is detected in the pituitary (Hill et al., 1992; Costa et al., 1993; Zogopoulos et al., 1996). Neural GH has also been shown to be bioactive in developing nervous tissues before it is secreted from the pituitary in rodents (Turnley et al., 2002; Ajo et al., 2003). For instance, GH promotes cell proliferation and cell diffentiation in cerebral cortical cells at E14 and E17, before GH is present in the pituitary gland (Ajo et al., 2003) and GH inhibits neuronal cell differentiation in E12.5 neuroepithelial cells in mouse, before pituitary differentiation (Turnley et al., 2002). Taken together, these studies strongly suggest GH acts as an autocrine / paracrine growth factor in nervous system development, although endocrine roles are likely to be involved perinatally and postnatally in adults.

1.2.5 GH: an autocrine / paracrine or endocrine in the development of the neural retina

Recent evidence suggests GH is present in and contributes to the development of the neural retina and related ocular tissues.

1.2.5.1 Sites of GH and GHR production during ocular development

As shown in Table 1.5 and 1.6, GH immunoreactivity is present in all major ocular structures in the early chick. Indeed, GH is detected in the whole eye, using western blotting, including the neural retina and the pigmented epithelium (Harvey et al., 2003). Within the eye, an immunoreactive signal is detected in the lens epithelium and fiber cells, in the developing cornea, sclera and choroid but also in cells of the developing retina, particularly in cells of the GCL in the neural retina, and in the retinal pigmented epithelium (Harvey et al., 2001; Harvey et al., 2003; Harvey et al., 2004). In perinatal rats, GH is detected in late embryonic eye and in retina from P0 to P21 (at constant levels) (Harvey et al., 2006a; Modanlou et al., 2006) and in the neonatal neural retina, GH is more specifically detected within the nucleus or cytoplasm of cells in the GCL and in the OFL (Harvey et al., 2006a). This immunoreactivity is likely to reflect local sites of synthesis. Indeed like GH protein, GH mRNA is present in the chick embryo whole eye before mid-embryogenesis; in the neural retina and in the retinal pigmented epithelium (Harvey et al., 2003; Harvey et al., 2004). Interestingly, Takeuchi and collegues (2001) could not amplify the full length GH cDNA in the late chick embryo. Instead, they discovered a new truncated GH isohormone (scGH) lacking exon 1 to 3 in the whole eye, although they could not detect it in any subocular tissues (Takeuchi et al., 2001). Harvey and collegues, on the other hand, could amplify GH with primers spanning exon 3 to 5 in the whole eye and retinal tissues of the second "trimester" chick embryo (Harvey et al., 2004). The putative presence of full length mRNA and of additional GH isohormones and variants need, therefore, to be clarified. In rodents, exon 1-5 GH mRNA is detected in the whole eye of late fetal rat and in the whole eye and neural retina of neonatal rats, particularly in the somata and proximal axons of cells in the GCL but also somewhat in cells of the developing inner nuclear layer adjacent to the inner plexiform layer (Harvey et al., 2006a).

Table 1.5. Prenatal and neonatal ocular GH mRNA distribution

Species	Tissue	Day	Reference
Chicken	Whole eye	E9	(Harvey et al., 2003)
		E15, 21	(Harvey et al., 2004)
Chicken	Retinal pigmented epithelium	E7	(Harvey et al., 2003)
		E9	(Harvey et al., 2004)
Chicken	Neural retina	E9	(Harvey et al., 2004)
Rat	Whole eye	E17, P0	(Harvey et al., 2006a)
Rat	Neural retina	P0	(Harvey et al., 2006a)

Table 1.6. Prenatal and neonatal ocular GH protein distribution

Species	Tissue	Day	Reference
Chicken	Whole eye	E7	(Harvey et al., 2003)
Chicken	Neural retina	E3	(Harvey et al., 2001)
		E6-9	(Harvey et al., 2003)
			(Harvey et al., 2004)
Chicken	Retinal ganglion cells	E8	(Sanders et al., 2005)
Chicken	Retinal pigmented epithelium	E7-8-9	(Harvey et al., 2001)
			(Harvey et al., 2003)
		E13-21	(Takeuchi et al., 2001)
Chicken	Lens	E6	(Harvey et al., 2001)
			(Harvey et al., 2003)
Chicken	Choroid layer	E7	(Harvey et al., 2003)
		E9	(Harvey et al., 2004)
Chicken	Sclera	E7	(Harvey et al., 2003)
Chicken	Cornea	E7	(Harvey et al., 2003)
Rat	Whole eye	E18	(Harvey et al., 2006a)
Rat	Neural retina	P0	(Harvey et al., 2006a)
Rat	Retina	P0-21	(Modanlou et al., 2006)
Rat	Vitreous	P0-21	(Modanlou et al., 2006)

GH is also detected in the vitreous humor in early embryonic chicks and in early postnatal rats (Sanders et al., 2003; Modanlou et al., 2006). In the early chick embryo, coimmunoprecipitation studies demonstrated that GH is associated with the proteoglycan opticin and together, these two molecules form a 60-62K complex within the vitreous humor (Sanders et al., 2003). In addition, GH and opticin colocalize in cells of the GCL (Sanders et al., 2003). It is thus speculated that opticin might act to sequester and concentrate GH in the vitreous and it might also regulate GH release from retinal and lens cells (Sanders et al., 2003). In early postnatal rats, GH is detected in the vitreous humor between P0-14 and its levels are decreased at P14-21 (Modanlou et al., 2006).
Ocular tissues are target sites of GH action (Table 1.7 and 1.8). The intracellular, extracellular and full length GHR mRNA are detected in the whole eye, neural retina and retinal pigmented epithelium in early chick embryos (Harvey et al., 2003), while GHR protein is detected in the optic vesicle and in the neural retina (Harvey et al., 2001; Harvey et al., 2003). In the fetal rat, GHR / BP immunoreactivity is strong in all retinal cells and it is also intense in E18 corneal epithelium, conjunctiva and lacrimal gland but less at E12-16 (Garcia-Aragon et al., 1992). Moreover in the P10 rat retina, GHR / BP immunoreactivity is present in the retinal ganglion cells with intense immunoreactivity, in bipolar cells and outer plexiform layer with strong immunoreactivty, and in the optic nerve fiber, the inner plexiform layer and bipolar cells with weak to moderate immunoreativity (Lobie et al., 1993).

1.2.5.2 GH action in retinal development

1.2.5.2.1 GH in retinal and optic pathway development

In adult teleost fish, intraperitoneally- injected recombinant trout GH promotes mitosis of progenitor cells in the neural retina within the ciliary germinal zone but also of inner nuclear layer progenitors, precursor of rod photoreceptors (Otteson et al., 2002). This action is thus likely to reflect an endocrine role for GH. Moreover, GHRs are expressed in the retina (Otteson and Hitchcock, 2003) and exogenous GH action on retinal neurogenesis is associated with the upregulation of retinal IGF-1, in addition to liver IGF-1 (Otteson et al., 2002). Retinal IGF-1 might thus mediate endocrine GH action locally.

Table 1.7. Prenatal and neonatal ocular GHR mRNA distribution

Species	Tissue	Day	Reference
Chicken	Whole eye	E7	(Harvey et al., 2003)
Chicken	Neural retina	E7	(Harvey et al., 2003)
		E17	(Takeuchi et al., 2001)
Chicken	Retinal pigmented epithelium	E7	(Harvey et al., 2003)
		E17	(Takeuchi et al., 2001)
Chicken	Choroid	E17	(Takeuchi et al., 2001)

Table 1.8. Prenatal and neonatal ocular GHR / BP protein distribution

species	Tissue	Day	reference
Chicken	Optic vesicles	E3	(Harvey et al., 2001)
Chicken	Neural retina	E8	(Harvey et al., 2003)
Chicken	Retinal ganglion cells	E8	(Sanders et al., 2005)
Rat	Retina	E18	(Garcia-Aragon et al., 1992)
		P10	(Lobie et al., 1993)

The normal development of the central and peripheral nervous systems requires a massive wave of programmed cell death or "apoptosis" (Hamburger and Levi-Montalcini, 1949; Cowan et al., 1984). Cells of the nervous system are thought to survive due to appropriate levels of growth factors released from the target cell, neighbouring neurons and glial cells (Cowan et al., 1984). GH is an anti-apoptotic growth factor, as it promotes cell survival in embryonic chick retina explants at E6 and E8 but not following GH injection directly into the eye cup, probably due to low naturally occurring cell death at this stage (Sanders et al., 2005). An antiapoptotic role for GH in the embryonic neural retina was demonstrated by the immunoneutralization of its endogenous GH *in ovo,* which increases by 3.5 to 5.5 fold the number of apoptotic cells, depending on the function-blocking antibody used (Sanders et al., 2005). Within the neural retina, endogenous GH prevents the cell death of RGC in culture, as GH withdrawal induces the number of cell death by almost 3 fold (Sanders et al., 2005). GH antiapoptotic signaling pathways in chick RGCs require Akt phosphorylation, decreased caspase-3 activation and expression, reduced caspase- and calpain-dependant PARP-1 (poly (ADP-ribose) polymerase-1) cleavage and decreased AIF-1 and caspase 3 expression (Harvey et al., 2006b; Sanders et al., 2006).

Congenital pituitary GH deficiency has also been associated with various retinal and optic nerve defects. Septo-optic dysplasia or "De Morsier syndrome" is a syndrome associated with hypopituitarism (and isolated GH deficiency in particular), optic nerve hypoplasia and the absence of the septum pellucidum, a midline brain structure (Hoyt et al., 1970; Mutz et al., 1984; Willnow et al., 1996; Collett-Solberg et al., 1998; Nanduri and Stanhope, 1999). Optic nerve hypoplasia is characterized by a poorly developed optic nerve, suggesting a role for GH in the development of the optic nerve possibly by preventing death of RGC and / or by promoting RGC axon growth during ontogeny. GH excess has also been detected occasionally in young children suffering from neurofibromatosis type 1, a genetic disorder associated with tumor growth along some nerves and abnormal skin pigmentation (Drake et al., 2000). Five cases have been reported about children with GH hypersecretion and optic pathway glioma (affecting the optic nerve, optic chiasm and / or optic tract) and accompanied in most cases by impaired vision (Costin et al., 1973; Crawford and Buckler, 1983; Duchowny et al., 1984; Manski et al., 1994; Drake et al., 2000) .

1.2.5.2.2 GH in the development of other ocular tissues

Congenital GH deficiency (GHD) is linked to ocular abnormalities affecting ocular tissues in addition to the neural retina. Children suffering from congenital GHD display a shorter ocular axial length and associated hyperopia (farsightedness), whether they undergo GH replacement therapy or not (Parentin et al., 2004). Congenital GHD children receiving GH therapy display an increase in ocular axial length over time and a change in refraction, but not as much as that observed in healthy subjects (Parentin and Perissutti, 2005).

GHD has also been associated with *retinis pigmentosa* in a rare syndrome referred to as RHYNS syndrome characterized by *retinitis pigmentosa*, hypopituitarism, nephronophthisis, and skeletal dysplasia (Di Rocco et al., 1997; Hedera and Gorski, 2001). Thus, case reports describe GHD children suffering from impaired vision associated with impaired optic nerve head, rods and cones physiology, retinal vasculature, retinal pigmentation (Di Rocco et al., 1997; Hedera and Gorski, 2001). GHD has been reported in one patient affected by Cockayne syndrome, a rare autosomal recessive disorder whose symptoms include pigmentary degeneration of the retina (Park et al., 1994) and in Jacobsen syndrome, whose symptoms include bilateral inferior colobomas (incomplete fusion of the choroid fissure) of the iris, choroid and retina, and CNS abnormalities (Pivnick et al., 1996).

In addition, GH might be involved in angiogenesis, as children with congenital GHD display blood vessels with lower retinal branching points compared to healthy children and regardless of GH replacement therapy (Hellstrom et al., 1999). Reduced retinal vascularization is also observed in children with Laron-type dwarfism (Hellstrom et al., 2002) and it has previously been reported that these patients display visuomotor impairement (Shurka and Laron, 1975). Conversely, GH has been associated with diabetic retinopathy and retinal neovascularization. For instance, type I diabetic patients suffering from retinopathy display an elevation of GH upon insulin or GHRH administration, compared to patients without retinopathy (Sharp et al., 1984; Kaneko et al., 1985). On the other hand, the prevalence of retinopathy in GH deficient patients suffering from diabetes is significantly reduced (Merimee, 1978; Alzaid et al., 1994). In addition, neonatal transgenic mice overexpressing a *GH* antagonist gene have reduced induced retinal neovascularization (Smith et al., 1997). Suppression of GH secretion via somatostatin analogs prevents the progression of (proliferative) diabetic retinopathy (Grant et al., 2000) and induced-neovascularization and blood vessel tufts in neonatal mice (Smith et al., 1997; Higgins et al., 2002). Interestingly, somatostatin analogs have been shown to directly prevents endothelial cell growth *in vitro* by acting directly on the retina (Davis et al., 2001). Somatostatin could thus act indirectly via circulating GH or directly on the retina (Grant and Caballero, 2005), possibly via local retinal GH.

1.2.5.2.3 GH action in the retina: endocrine vs autocrine / paracrine

Retinal pathologies associated with pituitary GH deficiency and excess suggests that GH might act either on the retina indirectly or directly on retinal GHR, although the presence of GHR in the developing human retina is still unknown. If endocrine GH acts directly on the retina, it must, axiomatically, cross the blood ocular barrier. The BOB is complete by term in human and ^{125}I-bGH does not cross the blood-retinal barrier after a 5 hour perfusion in bovine (James and Cotlier, 1983). However, the BOB has been shown to be leaky perinatally and its permeability is altered in cases of diabetes mellitus, *retinitis pigmentosa* and optic nerve disease, for instance (Cunha-Vaz, 2004). In addition in neonatal rats, vitreal GH levels are 10% that of serum in newborns, and vitreal and seral GH levels both decrease neonatally, suggesting vitreal GH might originate form the systemic circulation, at least in part (Modanlou et al., 2006). The possibility that endocrine GH promotes direct action is thus uncertain.

In perinatal rats, GHR / BP are detected in retinal cells, especially in RGCs in P10 rats, indicating that the retina is also a site of GH action in rodents (Garcia-Aragon et al., 1992; Lobie et al., 1993). GH might act as an autocrine in neonatal rats, especially as the distribution of GH mirrors that of GHR (GH is detected in cells of the GCL as well) and as GH is synthesized locally in cells of the GCL and in the inner plexiform layer (Harvey et al., 2006a). In addition, GH might act as a paracrine, since it is detected in neonatal rat vitreous (Modanlou et al., 2006) and possibly as an endocrine indirectly or directly via the BOB, provided it can cross it. GH is highly likely to promote retinal development in the early chick embryo in a paracrine / autocrine manner. Indeed, endogenous GH has been shown to prevent cell death in early embryonic chicks (Sanders et al., 2005). This survival role for GH reflects autocrine / paracrine action, especially as GH protein and mRNA, as well as GHR, are detected in the developing retina before GH is released into the systemic circulation (Harvey et al., 2001; Harvey et al., 2003; Harvey et al., 2004). In addition, the presence of GH in the retinal pigmented epithelium, cornea, and lens indicates extraretinal ocular GH might act as a paracrine on retinal GHR (Harvey et al., 2001; Harvey et al., 2003; Harvey et al., 2004).

Recent research has established that GH and GHR are distributed in the developing neural retina but also in other ocular tissues, and that GH promotes cell (including RGCs) survival and neurogenesis in the developing retina and in adult retinal stem cells (Otteson et al., 2002; Sanders et al., 2005). In addition, congenital deficiency in GH and GH resistance has been shown to induce a variety of ocular symptoms, including retinal- and optic nerve-associated pathologies, at least in human. Taken together, this strongly suggests GH is a key player in the development of the neural retina and in retinal ganglion cells in particular. In addition, GH is likely to act as an autocrine / paracrine, especially in early chick embryo, and as an endocrine. GH could also be present and act in the developing optic tectum, the RGC target in birds, as GH appears to be important in the nervous system development. GH is thus a hitherto unsuspected major growth factor involved in the development of the visual system.

1.3 Hypothesis and objectives

1.3.1 General hypothesis:

GH, conventionally, is considered to be an endocrine, released from pituitary somatotrophs into peripheral circulation, with actions at distant target sites (Harvey et al., 1995). However, GH is also locally produced in many extrapituitary tissues, where it acts as an autocrine / paracrine growth factor (Harvey and Hull, 1997). GH immunoreactivity has thus been detected within many developing nervous tissues of embryonic chicks, including the neural retina (Harvey et al., 2001).

The general hypothesis of this thesis research is therefore that the presence of GH within the neural retina of early embryonic chicks, before pituitary differentiation, suggests that GH acts as an autocrine / paracrine growth factor in developing tissues of the visual system.

1.3.2 Objectives:

1.3.2.1 GH: an autocrine / paracrine growth factor in visual tissues of the embryonic chicks?

If GH acts as an autocrine / paracrine growth factor within tissues of the visual system, then axiomatically GH and GHR protein and mRNA must be detected in these tissues and endogenous GH must display biological functions.

At the start of this thesis work, very little was known about the origin of the GH signal detected in the neural retina. The distribution of GH was therefore characterized and the possibility that the *GH* gene was locally expressed was examined.

As GH was discovered within axons of RGCs (chapter 3), GH immunoreactivity was traced throughout the length of these axons and within their main target, the optic tectum. The possibility that the neural retina, RGC axons and the optic tectum are GH target sites and that GH promotes RGC axon growth was also investigated.

1.3.2.2 scGH: translation and bioactivity within the neural retina

Takeuchi et al. (2001) recently discovered a new GH isohormone within the eyes of embryonic chicks. The possibility that this new transcript is translated, present in ocular tissues and has biological activity was examined.

As the putative primary structure of scGH is severely truncated, the possibility that it might not be able to bind GH receptor was theoretically investigated.

1.3.2.3 GH in the mouse neural retina: distribution and action

The presence of GH and GHR in the retina of neonatal rats (Harvey et al., 2006a) suggests that GH could also promote retinal development in this species. A screening for GH actions in newborn mouse was therefore undertaken using GHR knockout animals and a proteomics approach.

1.4 Tissue and animal models

The chick embryo neural retina is the model of choice to study autocrine / paracrine GH in the developing visual system.

The neural retina has a relatively simple cytoarchitecture, compared to other tissues of the CNS, with a laminated structure composed of a small number of cell types whose morphology and topographical location are easily characterized (Mey and Thanos, 2000). The neural retina constitutes, therefore, a very appropriate tissue to study GH roles in the developing nervous system.

The chick embryo has been one of the favored animal models to study vertebrate developmental biology for more than 2000 years (Stern, 2005). It is easily accessible and manipulated within the shell ("*in ovo*") (Brown et al., 2003), whereas rodents are not accessible *in utero*. Moreover in some cases, chicken have been favored over rodents to study human ocular development (Lawrence and Azar, 2002; Reza and Yasuda, 2004). The chick embryo eye is functional at the time of hatching while, contrary to humans, rodents are altricial species and as such, their eyes are not open or fully developed at birth.

In addition, the chick embryo appears to be the model of choice to study autocrine / paracrine GH's role in the developing neural retina. Most neural retina development occurs in the absence of endocrine GH while it occurs in the presence of local GH, which is detected as early as E3 in this tissue (Harvey et al., 2001). Indeed, the development of the neural retina is essentially complete before pituitary GH is secreted into the circulation. In particular, RGC axon growth and cell death are complete by E16 (Mey and Thanos, 2000), one day before pituitary GH is detected in the circulation (Harvey et al., 1979). In addition, the presence of the blood ocular barrier in the chick eye is functional at E16 (Liebner et al., 1997) and might preclude circulating GH from penetrating after E17. Local GH, therefore, and NOT endocrine GH, is highly likely to promote the neural retina development of early chick embryos. In contrast, similar developmental period of the neural retina occurs much later in rodents, shortly before and after the pituitary GH is secreted into the systemic circulation. The neural retina could thus be responsive to direct or indirect actions of endocrine GH in addition to autocrine / paracrine GH. These two modes of action cannot, therefore, be distinguished in these species. Rodents can, however, be genetically modified and this advantage was exploited to study GHR mediated roles using GHR knockout neonatal mice.

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Chapter 2

Retinal Growth Hormone in the Chick Embryo

Chapter 2: Retinal Growth Hormone in the Chick Embryo[1]

2.1 *Introduction*

It is now well established that GH immunoreactivity is present in the brain, spinal cord, and some peripheral nerves (1, 2). In recent studies, widespread GH immunoreactivity was also seen in the neural retina of embryonic chicks (3). Because exogenous GH has been shown to stimulate retinal development in the teleost eye by inducing retinal IGF-1 synthesis (4), retinal GH may have roles in the development of the eye or ocular function.

In the chick embryo, the GH immunoreactivity found in the neural retina (3) was detected by three different antibodies raised against chicken GH and was lost after the preabsorption of the antibodies with excess recombinant GH. Shortly afterward, Takeuchi *et al* (5) identified a novel GH mRNA transcript in the eye of embryonic chicks that coded for a truncated (16.5 kDa) protein. These authors found GH immunoreactivity, cross-reacting with antibodies against monomer (approximately 22 kDa) rat GH, in the retinal pigmented epithelial (RPE) cells but not in the neural retina. The GH immunoreactivity in the RPE was, however, associated with a large protein with a molecular mass of 80-86 kDa, even when examined under reducing conditions. The novel GH mRNA transcript identified by Takeuchi *et al* (5) was, furthermore, not present in the RPE, neural retina, sclera, lens, vitreous body, iris, ciliary body, cornea, or rectus muscle. Takeuchi *et al* (5) were also unable to find expression of the full-length GH mRNA in the eye, as found in the pituitary gland. These paradoxical findings by Takeuchi *et al* (5) are difficult to reconcile and conflict with our original observations on the presence of retinal GH immunoreactivity (3). The expression and cellular localization of GH in retinal tissues has, therefore, been further assessed in the present study.

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[1] A version of this chapter has been published: Baudet ML, Sanders EJ and Harvey S. Retinal growth hormone in the chick embryo *Endocrinology*. 2003 **144**: 5459-68. Dr. Sanders provided the Western blots and immunohistochemical photomicrographs used in the publication although identical results were subsequently achieved independently. Dr. Xin-Wei Lin was responsible for the real-time PCR.

2.2 Materials and Methods

Animals and tissues

Fertile White Leghorn eggs from the University of Alberta Poultry Unit (Shaver White strain) were incubated at 37.5 °C in humidified air (3). The eggs were rotated one-quarter of a revolution each day during incubation. The eyes from decapitated embryos were collected in PBS (pH 7.4) and, in some cases, the neural retina was dissected out. Embryos at embryonic day (E) 7-8 were used because we have previously found GH immunoreactivity in the eyes of E7 and E8 embryos (3) before the ontogenic differentiation of pituitary somatotrophs (at approximately E12) (6).

Retinal GH mRNA

RT-PCR. The presence of GH mRNA was assessed using RT-PCR. Total RNA was extracted from the eyes of neural retinas of E7 embryos using Trizol reagent (Invitrogen, Carlsbad, CA) and any contaminating genomic DNA removed by DNase1 treatment (Ambion RNA Diagnostic, Austin, TX). DNA-free total RNA was then reverse transcribed using 15 U Thermoscript (Invitrogen) in the presence of 2.5 μM Oligo(dT)20, deoxynucleotide triphosphate mix (1mM), cDNA synthesis buffer (1X), dithiothreitol (5mM), and 40 U RNase OUT (Invitrogen). The mixture was denatured (5 min at 65 °C) and then incubated at 59 °C for 1 h. The reaction was then terminated by a 5-min incubation at 85 °C, and the template RNA was removed from the newly synthesized cDNA by RNase H digestion (Invitrogen; 20 min at 37 °C). For comparative purposes, reverse-transcribed RNA from slaughterhouse (42 d old; Hubbard/Ross strain) chicken pituitary glands was used as a positive control.

Tissue RNA, not reverse transcribed (in the absence of Thermoscript), served as a negative control. Aliquots (1.0 μl) of the cDNAs were then amplified in the presence of oligonucleotide primers (DNA Core Facility, University of Alberta) for the chicken pituitary GH cDNA (7). Oligonucleotide primers PE4F (sense) (5) and PE5R (antisense) (5) were used to amplify a 360-bp fragment derived from most of exons 4 and 5 (Fig. 2.1). Primers CLR1 (sense) (8,9) and CLR2 (antisense) (8,9) were used to generate a 690-bp full-length chicken GH cDNA (Fig. 2.1). The primers (10 pmol each) were in the presence of excess deoxynucleotides (0.2 mM of each), 10X PCR buffer, 1.5 mM $MgCl_2$, 50 mM KCl and 2.5 U *Thermus aquaticus* DNA polymerase PCR beads (PURE Taq

Ready-To-Go PCR Beads, Amersham, Buckinghamshire, UK). The reaction mixtures were then denatured at 94 °C (5 min) and subjected to 35 cycles of denaturation (94 °C for 30 sec), annealing (55 °C for 30 sec), and extension (72 °C for 30 sec), with a final extension of 3 min at 72 °C, in a thermal cycler (Tech Gene, Fisher Scientific, Edmonton, Alberta, Canada). The amplified cDNAs were then electrophoresed in ethidium bromide-stained agarose gels (1.2% wt/vol) and viewed under UV light, and compared with DNA molecular weight markers.

Cloning. Total RNA from E7 neural retina was reverse transcribed and cDNA obtained. PCR was then performed as above with oligonucleotide primers CLR1 and CLR2 using platinum high-fidelity *Taq* DNA polymerase (Invitrogen) because it has proofreading activity. The 690-bp PCR product was then inserted into the pCR II-TOPO vector (Invitrogen), which was then transformed into chemically competent *Escherichia coli* (Invitrogen) and the cells allowed to grow overnight at 37 °C. The vectors were then collected using a HiSpeed MidiKit (Qiagen, Mississauga, Ontario, Canada) and the insert obtained by EcoRI endonuclease digestion and visualized on ethidium bromide-stained agarose gels. The sequence of the insert was determined by the University of Alberta DNA Core Facility.

In situ hybridization

Probe synthesis. The 690-bp full-length pituitary GH cDNA obtained by PCR using primers CLR1 and CLR2 was subcloned into the pCR II-TOPO vector (Invitrogen), as before. The vector was then linearized with restriction endonucleases HindIII and NotI for the subsequent production of antisense and sense probes, respectively. Digoxigenin (DIG)-labeled antisense and sense riboprobes were synthesized by *in vitro* transcription with T7 and SP6 RNA polymerase, respectively, and DIG RNA labeling mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Probe concentration was determined by dot blot analysis against a DIG-labeled RNA standard.

Hybridization procedure. E7 chick embryo heads were fixed overnight at 4 °C in 4% paraformaldehyde in PBS, dehydrated, and embedded in paraffin. Tissue sections were obtained, deparaffinized in Citrisol (Decon Laboratories, Bryn Mawr, PA), rehydrated through a graded ethanol series, and washed in diethylpyrocarbonate (DEPC)-treated water and then in DEPC-PBS. The sections were then incubated in fresh 0.1% DEPC-

PBS (2 x 15 min) to remove RNase, treated with proteinase K (10 μg/ml, 10 min, 37°C), postfixed in 4% paraformaldehyde (10 min), and incubated in 0.1 M triethanolamine (pH 8.0) for 10 min. The sections were then prehybridized with 50% formamide / 2x saline sodium citrate (SSC) (30 min, 60 °C) and hybridized in a moist chamber (overnight, 60 °C) with hybridization solution (50% formamide, 2x SSC, 250 μg/ml tRNA, 50 μg/ml heparin, 2% blocking solution, and sense or antisense DIG-labeled riboprobes at concentrations of 2 μg/ml that had previously been denatured (5 min, 80 °C). The sections were sequentially washed in 2x SSC (10 min), 2x SSC / 50% formamide (45 min, 45 °C), 2x SSC (3x15 min), 2x SSC (30 min, 37 °C), 0.1x SSC (60 min, 60 °C). The sections were then washed (2x5 min) in buffer one (0.01 M Tris, 0.15 M NaCl, pH 7.5), and immunohistochemical detection of the hybridized probe was achieved by incubation (2h) with an anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics) in 1% blocking solution (Roche Diagnostics). The sections were then washed in buffer one (2x15 min), equilibrated (5 min) in buffer two (0.1 M Tris, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5), and incubated in buffer two containing a commercial substrate for alkaline phosphatase [5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium chloride; Roche Diagnostics] until color developed. The color development was stopped by washing in Tris-EDTA buffer (2x5 min). The sections were then rinsed in PBS (3x5 min) and mounted with an aqueous medium. Tissue sections from the pituitary glands of slaughterhouse (42 d) chickens were similarly treated, although at probe concentrations of 200 ng/ml, and the labeling of somatotrophs in the caudal lobe (Fig. 2.2) provided a positive control (10,11) for comparison.

Retinal GH immunoreactivity

Western blotting. Tissues were collected into a protease inhibitor solution [HEPES, $MgCl_2$, EDTA, EGTA, aprotinin, leupeptin, and pepstatin in 1% (wt/vol) phenylmethylsulfonyl fluoride] and homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). After centrifugation and protein determination, by the Bradford dye-binding procedure (Bio-Rad Laboratories, Mississauga, Ontario, Canada), the supernatants were stored at -20° C before Western analysis. Samples (containing 20-50 μg protein) were analyzed by one-dimensional SDS-PAGE (8, 9). The samples were added to loading buffer (10% (wt/vol) glycerol, 5% (wt/vol) β-mercaptoethanol, 2% sodium dodecyl sulfate, 0.001% (wt/vol bromophenol blue, pH 6.8), and 10% (wt/vol) dithiothreitol and denatured at 100 °C for 4 min and the proteins separated by electrophoresis in 15% gels. After electrophoresis, the proteins were equilibrated in transfer buffer (25 mM Tris, 192 mM Glycine, 20 % (wt/vol) methanol) and transferred electrophoretically (100 V for 1 h at 4 °C) to nitrocellulose paper (Bio-Rad Laboratories). After transfer, nonspecific binding sites were blocked with 5% (wt/vol) nonfat dry milk in Tris-buffered saline (25 nM Tris HCI, 0.5M NaCl, pH 7.6) containing Tween 20 for 1 h at room temperature. GH immunoreactivity was detected using a polyclonal antibody raised in rabbit against chicken pituitary (α-cGH-1) (13), diluted 1:3000 in Tris-buffered saline/5% (wt/vol) nonfat dry milk. After an overnight incubation at room temperature, the membranes were washed with Tris-buffered saline containing Tween 20 (3x10 min) and then incubated for 1 h at room temperature with a biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA; 1:500). Antibody binding was then visualized using ABC reagents (Vector Laboratories) 1 h at room temperature, and after washing, the blots were developed with an enhanced chemiluminescence detection system (ECL kit, Amersham) and exposed to X-AR film (Kodak, Rochester, NY).

ELISA. The presence of GH immunoreactivity in retinal tissue extracts (n = 3) was investigated using an indirect ELISA, similar to that described by Martinez-Coria *et al* (12). Briefly, 96-well microtiter plates (Immulon 2HB, MTY Labs, Inc., Vienna, VA) were coated overnight at 4 °C with 12 ng recombinant chicken GH (Amgen, Thousand Oaks, CA) in 100 μl 1 M carbonate buffer (pH 10.3). Tissue extracts or serial dilutions of recombinant chicken GH (5.0-5120 ng/ml) in TPBS [0.01 M sodium phosphate, 0.15 mM NaCl, 0.05% (wt/vol) Tween 20, 1% (wt/vol) nonfat dry milk] were then incubated for 16 h with 100 μl primary antibody (at a final concentration of 1:50,000) raised in rabbit against native pituitary GH (α-cGH-1) (13). This antibody is specific for GH and has no cross-reactivity with any other chicken pituitary hormone (13). The samples and standards (100 μl) were then added to the coated wells and incubated for a further 2 h at room temperature. Horseradish peroxidase conjugated to anti-rabbit IgG (Bio-Rad Laboratories) was then added [at a dilution of 1:3000 in 5% (wt/vol) nonfat dry milk, in 0.01 M TPBS, pH 7.0] and incubated for 2 h at room temperature. Bound secondary antibodies were then developed by reaction with 2,2'-amino-di-[3-ethylbenzothiazoline sulfonate] substrate (Roche, Mannheim, Germany). The plates were read 1 h later in an automatic ELISA microplate reader (Bio-Rad Laboratories) at a wavelength of 405 nm. The assay had sensitivity of 2 ng/well (20 ng/ml) and inter-assay and intra-assay coefficients of variation less than 4%.

Immunohistochemistry. Tissues were fixed in freshly prepared paraformaldehyde (4% wt/vol; Sigma, Mississauga, Ontario, Canada) overnight at 4°C. They were then dehydrated in a graded series of ethanol (50%, 15-30 min; 70%, 30-60 min; 95%, 30-120 min) and cleared with Hemo-De (Fisher Scientific) for 30 min. Tissues were then infiltrated with paraffin wax for 24-48 h at 60°C under normal atmospheric pressure. Serial transverse (4-8 μm) sections were taken using a microtome and mounted onto charged slides (Fisher Scientific). Confocal immunocytochemical staining was performed with a specific polyclonal antibody raised in rabbit against native chicken GH (α-cGH-1) (13) and diluted 1:500 in 4% BSA for 1.5 h at room temperature. After incubation, the slides were washed three times for 10 min in 4% BSA in PBS. Sections were then incubated for 1 h at room temperature in goat anti-rabbit IgG conjugated to fluorescein isothiocyanate at a dilution of 1:100. Retinal ganglion cells (RGCs) were identified using the anti-islet mouse monoclonal antibody, 39.4D5 (14, 15) obtained from the Development Studies Hybridoma Bank at the University of Iowa. Sections were labeled with undiluted 39.4D5 antibody overnight at 4°C and then incubated with a goat antimouse IgG conjugated to AlexaFluor (Molecular Probes Inc., Eugene, OR), at a dilution of 1:200 for 1.5 h at room temperature. The blocking agent was 4% BSA and sections were washed three times for 10 min each, with BSA in PBS between each step. The labeled sections were examined using a LSM 510 confocal microscope (Carl Zeiss, Göttingen, Germany) equipped with appropriate lasers. Controls, in which the antibodies were replaced by nonimmune serum, were negative.

Retinal GH action: GH-induced IGF-1 expression

A role for GH in retinal development has been suggested by its stimulation of IGF-1 synthesis in the teleost eye (4). The possibility that GH may similarly act in the neural retina of early chick embryos was therefore investigated.

Neural retinas were dissected from the eyes of individual E8 embryos into Tyrode's saline and explanted into culture dishes coated with type I collagen, with the neural retina in contact with the substratum. The collagen (Vitrogen 1007; Cohesion Corp., Palo Alto, CA) was prepared by mixing it in the ratio 8 parts collagen:1 part medium 199 (Life Technologies, Inc., Gaithersburg, MD), 10 times concentrated:1 part 0.1 M sodium hydroxide, and allowing it to gel in the dishes at 37°C for 1 h. Pieces of retina were placed on the collagen, spread out, and allowed to adhere at 37°C for 3 h. Medium 199, plus antibiotics and 10% fetal bovine serum were then added, containing 10^{-6} M chicken GH (Dr. A. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases' National Hormone and Pituitary Program, Torrance, CA). This dose was used because the maximal production of IGF-1 by chicken hepatocytes requires *in vitro* GH concentrations of approximately 1-10 µg/ml (16). Control samples were cultured in the absence of GH.

After 48 h, total RNA was extracted from the cultured, retinal tissues using Trizol reagent (Invitrogen). Before cDNA synthesis, the total RNA was treated with DNase I (amplification grade, Invitrogen) to eliminate contamination with genomic DNA. cDNA was synthesized from 1 µg total RNA using Superscript II RNase H-reverse transcriptase (Invitrogen) with a mixture of oligo(dT)12-18 and random primers (Invitrogen). cDNA was then amplified in a thermal cycler in the presence of oligonucleotide primers (IGF-1; forward primer: TTCTTCTACCTGGCCTGTG; IGF-1-2 reverse primer: CATACCCTGTAGGCTTACTG) designed to generate a 147-bp fragment of the chicken IGF-1 cDNA sequence (M33791, Gene Bank) (17). The PCR involved an initial denaturation for 10 min at 95°C, followed by 38 cycles of PCR amplification with denaturation for 30 sec at 95°C, annealing for 30 sec at 55°C, and extension for 30 sec at 72°C. After PCR, the reaction products were visualized on ethidium-bromide agarose gels. mRNA that had not been reverse transcribed served as a negative control. The same PCR conditions were used for real-time PCR to quantify IGF-1 mRNA, using an iCycler thermal cycler attached to a iCycler IQ real-time PCR detection system (Bio-Rad Laboratories) with 96-well 0.2 ml thin-walled PCR plates (Bio-Rad Laboratories). PCR components were made from SYBR Green PCR Cone reagents (PE Applied Biosystems, Foster City, CA) and Ampli Taq Gold DNA polymerase (PE Applied Biosystems) according to the manufacturer's protocol. A standard curve was constructed from cDNA synthesized from different amounts of DNase 1-treated total RNA (50-5000 ng). For an internal control, real-time RT-PCR was also performed using a primer set (18S-1: TTCGTATTGTGCCGCTAGAG, and 18S-2: GCATCGTTATGGT CGGAAC) designed to generate a 154-bp fragment of chicken 18S rRNA (Gene Bank accession no AF173612) (18). The PCR involved an initial denaturation of 10 min at 95°C, followed by 20 cycles of PCR amplification with denaturation for 3 sec at 95°C, annealing for 30 sec at 53°C, and extension for 30 sec at 72°C. All the primers were synthesized by the University of Alberta DNA Core Facility. The concentrations of IGF-1 mRNA and 18S rRNA in each sample were determined by the iCycler IQ real-time PCR detection system, and the concentrations of IGF-1 mRNA were then normalized relative to the 18S rRNA concentrations. Statistical differences in the data were determined by Students *t* test.

2.3 Results

Retinal GH mRNA

RT-PCR. As expected (5), a 360-bp cDNA was generated from reverse-transcribed mRNA from the whole eyes of E7 embryos, with oligonucleotide primers PE4F and PE5R (Fig. 2.3). This cDNA moiety was also generated, more strongly, with reverse-transcribed mRNA from the neural retina of E7 embryos (Fig. 2.3). This moiety was not present in the mRNA controls (Fig. 2.3).

Using the oligonucleotide primers CLR1 and CLR2, designed to amplify the full-length transcript of the pituitary GH cDNA, a 690-bp cDNA was generated with reverse-transcribed mRNA from the neural retinas of E7 embryos, identical in size to that generated from pituitary mRNA (Fig. 2.4). This cDNA was not generated in the negative controls (Fig. 2.4). After cloning, the nucleotide sequence of this cDNA was 99% homologous (689/690 bases) to the published sequence for chicken pituitary GH cDNA (7) (Fig. 2.5). The only difference was a base substitution at position 588 (623 of the cDNA fragment) (an A for G substitution). This substitution would not change the amino acid (lysine) sequence of the predicted protein, which was 100% identical with chicken pituitary GH (7). In comparison with the sequence reported by Tanaka *et al* (7), the pituitary GH cDNA reported by Lamb *et al* (19) differs by base pair substitutions at positions 156 (T for C), 165 (C for T), and 302 (C for T). The pituitary GH cDNA reported by Baum *et al* (20) differs by base pair substitutions at positions 336 (G for C), 465 (A for C), 466 (T for A), 467 (C for G), 468 (A for C), and 504 (T for C). The pituitary GH cDNA reported by Zhvirblis *et al* (21) differs by base pair substitutions at position 336 (G for C) and 339 (T for C). None of these substitutions was present in retinal GH cDNAs sequence from six separate clones.

In situ hybridization. Hybridization of the full-length GH antisense probe was widespread in the neural retina, but it was particularly intense in large cells in the RGC layer (Fig. 2.6A). The cytoplasmic distribution was clearly visible at higher magnification (arrows, Fig. 2.6B-D). GH mRNA was also present in axons emanating form RGC soma (arrowhead, Fig. 2.6D) but was absent from the OFL composed of RGC axons (Fig. 2.6A-C). No labeling was seen when the antisense riboprobe was replaced by the sense riboprobe (Fig. 2.6E).

Retinal GH immunoreactivity

Western blotting. GH immunoreactivity in extracts of the pituitary gland was mainly associated with proteins of 22 and 25 kDa and with proteins of 15 and 16 kDa (Fig. 2.7). In contrast, although the 15 and 16 kDa GH-immunoreactive proteins were abundantly present in extracts of E6, E8, and E9 neural retinas, only trace amounts of the 22 and 25 kDa proteins were present in these tissues (Fig. 2.7). GH immunoreactivity was also present in the vitreous humor of E6, E8 and E9 embryos, although this was associated with the 15 kDa protein (Fig. 2.7). The GH-immunoreactive 15 kDa protein was also present in medium in which the neural retinas of E6, E8, and E9 had been cultured for 3 d (Fig. 2.7). Other GH moieties of 16, 18, 22 and 25 kDa were also present in the culture medium but in lesser amounts (Fig. 2.7). When the neural retinas from the eyes of E8 embryos were cultured for 24 h, the 15 kDa protein was the only GH-immunoreactive band detected in the culture medium (Fig. 2.7).

After hatch, proteins with GH immunoreactivity of 15 and 16 kDa were present in the neural retinas of 1-, 5-, and 12-d neonates, in which immunoreactive proteins of approximately 25 kDa were also visible at 1 and 12 d (Fig. 2.8). By 42- d of age, the 15 and 16 kDa GH-immunoreactive proteins were no longer present in the neural retina, although a faint 25 kDa protein band was observed in this tissue. A GH-immunoreactive protein of 15 kDa was, nevertheless, abundantly present in the vitreous humor of 42-d-old chicks (Fig. 2.8).

ELISA. Using ELISA, the estimated GH concentration in the neural retina of E7 embryos was approximately 0.245 μg/mg protein. The GH concentration in the pituitary gland of 42-d-old birds was, for comparison, 39.59 ± 2.8 μg/mg protein (n = 3).

Immunohistochemistry. GH immunoreactivity (*green fluorescence*) was widespread in the neural retina of E8 embryos (Fig. 2.9B), although it was most intense in a band of large cells located in the presumptive RGC layer. Double-labeled confocal microscopy, using a RGC-specific monoclonal antibody (39.4D5, *red fluorescence*; Fig. 2.9A), confirmed the localization of GH primarily within the RGCs (*yellow image*; Fig. 2.9C).

Retinal GH action: GH-induced IGF-1 expression

RT-PCR amplified a 147-bp fragment of the chicken IGF-1 cDNA from reverse-transcribed retinal mRNA after E8 retinas had been cultured for 48 h in Medium 199 (Fig. 2.10A). When cultured in 10^{-6} M chicken GH for 48 h, the amount of IGF-1 mRNA detected was markedly increased (Fig. 2.10A), more than 4-fold ($P < 0.01$, Student's test), when quantified by real-time PCR (Fig. 2.10B).

2.4 Discussion

Although GH gene expression primarily occurs postnatally in the pituitary gland, GH mRNA has been detected in the placenta, mammary gland, ovary, testis, lung, liver, skeletal muscle, cartilage, teeth, skin, thymus, spleen, Peyer's patches, tonsils, salivary glands, and lymph nodes and is present in circulating lymphocytes and in blood endothelial and smooth muscle cells (22-29). This is, however, the first demonstration of a full-length GH mRNA in the neural retina.

The GH transcript identified in these studies hybridized with oligonucleotide primers CLR1 and CLR2 and the primer set PE4F and PE5R to generate cDNA fragments of predicted size (7), identical with those generated from reverse-transcribed pituitary GH mRNA. Moreover, apart from a single base substitution, the amplified full-length transcript was nearly identical with the published sequence of pituitary GH cDNA and coded for the same protein (7). These results therefore clearly demonstrate expression of the full-length GH gene in the neural retina of embryonic chicks. This finding is also consistent with the presence of the full-length GH transcript in the brain (8), thymus, spleen and Bursa of Fabricius (9), and testes (30) of chickens. These results conflict, however, with the recent findings of Takeuchi *et al* (5).

In contrast with our results, Takeuchi *et al* (5) were unable to generate a full-length GH cDNA from reverse-transcribed mRNA from embryonic (E17) chick eyes. These investigators were, however, able to generate a 360-bp cDNA from whole-eye mRNA using oligonucleotide primers PE4F and PE5R that spanned exons 4 and 5 of the pituitary GH gene. Although we were also able to generate this cDNA moiety with reverse-transcribed mRNA from the whole eye and from the neural retina of E7 embryos, Takeuchi *et al* (5), paradoxically, were unable to generate this moiety using reverse-transcribed mRNA from the retina, RPE, choroid, lens, sclera, vitreous body, iris, ciliary body, cornea, or the rectus muscle. They, therefore, thought that the GH transcript detected in the whole eye might have been derived from peripheral blood cells or other extraocular tissue collected when the eyes were removed from the embryos, rather than from the eye itself. It is, however, unclear how this transcript could be the source of the GH immunoreactivity that they found to be present in the RPE of their embryonic chicks.

Although the detection of this 360-bp cDNA indicates the presence of exons 4 and 5 sequences of the pituitary GH gene, Takeuchi *et al* (5) claimed, using 5' rapid amplification of cDNA ends, that this cDNA was derived from a novel GH transcript, small chicken GH. The cDNA for this transcript was reported to be 569 bp and composed of two exons, the first exon extending from the middle of intron C of the pituitary GH gene to the end of exon 4, with the second exon identical with exon 5 of the pituitary GH gene. It is, however, pertinent that the 5' rapid amplification of cDNA ends data presented by Takeuchi *et al* (5) does show the presence of larger cDNA moieties of approximately 700-900 bp, which may have been derived from the full-length transcript. These larger moieties were, however, not sequenced. Thus, although we did not find any evidence for the presence of the novel GH transcript reported by Takeuchi *et al* (5), more than one GH transcript may be present in the eyes of embryonic chicks. It is also possible that this difference may reflect a strain difference because the birds used by Takeuchi *et al* (5) were broilers (Rock Cornish), whereas ours were from a Leghorn strain (Shaver White). GH gene polymorphisms in intron and exon sequences have been reported for different chicken strains (31-33), and the single base pair substitution we found at position 588 of the GH cDNA is likely to reflect a similar polymorphism. This base pair substitution was similarly present in the GH cDNA cloned from the testes of this strain (30).

It is of interest that our nucleotide sequence for retinal GH is more homologous (99.9%) to the pituitary GH cDNA sequence reported by Tanaka *et al* (7) than other reported chicken pituitary GH cDNAs, because the pituitary GH cDNA sequence reported by Baum *et al* (20) differs by six nucleotides (99.1% homology), the sequence reported by Lamb *et al.* *(19)* differs by three nucleotides (99.6% homology), and the sequence reported by Zhvirblis *et al* (21) differs by two nucleotides (99.7% homology). The predicted amino acid sequence of the protein coded by the retinal GH cDNA would, moreover, be 100% homologous with the protein coded by Tanaka's (7) pituitary GH cDNA, whereas the base pair substitutions reported by Baum *et al* (20), Lamb *et al* (19), and Zhvirblis *et al* (21) would result in proteins that differed by one amino acid (99.5% homology in each case).

The presence of the full-length GH mRNA in the neural retina and eyes of our embryonic chicks strongly supports the possibility that these tissues are extrapituitary sites of GH synthesis. Moreover, because pituitary somatotrophs do not differentiate in the chick embryo until at least E12 (6) and because immunoreactive GH is not present in peripheral plasma until E17 (6, 34), the presence of GH immunoreactivity in the eyes of embryonic chicks is likely to reflect its local production.

In our study, GH immunoreactivity was found throughout the neural retina of E7-8 embryos, but it was most intense in the RGCs. These results confirm the widespread presence of GH immunoreactivity in the E7 chick retina previously reported by Harvey *et al* (3). Takeuchi *et al* (5) also found GH immunoreactivity by immunohistochemistry in the eyes of chick embryos but only in the RPE and only between E10 and E20 (the RPE did not stain for GH immunoreactivity on E7, E8, or E9 or in 1- or 7-d-old neonates). The differences between these results are thus difficult to explain, but they are unlikely to reflect age-related differences in the embryos used. The antibody used by Takeuchi *et al* (5) was, however, raised against rat GH, whereas ours was raised against GH purified from chicken pituitary glands (13). Although both antisera readily detect monomeric GH in the pituitary gland, it is possible that these antisera recognize different epitopes and GH variants, and that these may be differentially located in tissues of the eye or differ in their temporal expression. Indeed, it is now well established that GH in the chicken pituitary is markedly heterogeneous with numerous size and charge variants that result from differences in posttranslational processing, proteolytic cleavage, and aggregation (35, 36). It is also pertinent that we have previously found that peripheral plasma GH concentrations in chickens measured by our homologous RIA poorly correlate with those measured using a rat GH RIA, which lacks sensitivity and specificity (37).

Although we found full-length GH cDNA in the eyes of embryonic chicks, the coded full-length protein was not readily detectable in Western blots of embryonic neural retinas, although GH immunoreactive proteins of approximately 25 kDa were present in the neural retina during neonatal and juvenile development. Smaller GH-immunoreactive proteins of 15 and 16 kDa were, however, abundantly present in the neural retina throughout embryonic development and in 1- to 12-d neonates. These smaller moieties and an 18 kDa protein are also present in pituitary extracts but in lesser amounts (35). It is, therefore, pertinent that the relative abundance of these moieties in the pituitary glands of chicken embryos is far greater than in neonates and adults (35). These moieties are likely to result from the proteolytic cleavage of monomeric GH (38). Indeed, a 15 kDa immunoreactive GH protein is derived from recombinant 25 kDa GH after its digestion with thrombin (38). This thrombin cleaved 15 kDa moity has recently been shown to promote bovine endothelial cell proliferation and to inhibit the activity of hepatic deiodinase type III in chicken (38). These results of our study therefore indicate that ontogenetic changes in the posttranslational processing of the full-length protein might occur in the neural retina during development.

The antibody used by Takeuchi *et al* (5) did not detect any small molecular weight GH moieties in the pituitary glands or ocular tissues of neonatal chicks. Although these authors claimed to have identified a novel GH transcript in the embryonic eye that coded for a 16.5 kDa protein, they could not provide any evidence of its presence in these tissues. Although we, paradoxically, detected a 16 kDa GH moiety in the neural retina of embryonic chicks, we did not detect a truncated GH transcript. The 16 kDa GH moiety that we found could, however, provide evidence of a small chicken GH coded by the truncated transcript identified by Takeuchi *et al* (5) because it was not found in vitreous humor or the media used for the culture of retinal tissue. It is, therefore, of interest that the protein coded by the novel transcript identified by Takeuchi *et al* (5) did not have a signal sequence, and hence it was not thought to be a secreted protein.

In our study, the vitreous humor contained abundant GH immunoreactivity. This is a novel finding and suggests that it may act as a conduit for retinal GH acting at extra-retinal sites (possibly the lens, iris, ciliary body, vitreous body, or cornea). It may, alternately, act as a reservoir for retinal GH because GH was still present in the vitreous humor of juvenile chicks in which it had disappeared from the neural retina. This possibility is supported by the GH concentration in the vitreous being greater than in the neural retina, as indicated by the relative amounts of protein loaded on the gels. Moreover, because the 15 kDa GH moiety was abundantly present in the media cultured neural retinas, it is highly likely that the 15 kDa GH immunoreactivity in the vitreous humor is derived from the tissue. Our inability to detect GH immunoreactivity in the neural retinas of juvenile (42 d) birds further indicates ontogenetic changes may occur in the transcription or translation of GH in this tissue. This is similar to the ontogenic differences in the GH immunoreactivity of the RPE reported by Takeuchi *et al* (5).

The finding of GH immunoreactivity in the neural retina of embryonic chicks suggests roles for GH in ocular function, especially because GH receptor immunoreactivity has been similarly localized (3). This possibility is supported by our demonstration that GH markedly induces IGF-1 expression in the cultured neural retinas of E8 embryos. Increased IGF-1 production is the hallmark of GH action (4, 39) and a critical step in the intracellular cascade involved in GH signal transduction (40, 41). Because IGF-1 has established antiapoptotic roles that promote cell survival (42), particularly during neurogenesis of the chick neural retina (43-46), our results suggest GH involvement in these developmental processes. Indeed, exogenous GH has been shown to stimulate retinal development in the teleost eye by inducing local IGF-1 synthesis and the proliferation of stem cells and progenitors (4). GH is also thought to similarly induce neurogenesis in the developing brain and spinal cord (47, 48). It may also induce retinal angiogenesis because GH deficiency in humans is associated with reduced retinal vascularization (49), whereas exogenous GH promotes retinal angiogenesis (50), and GH excess is associated with retinopathy (51-53).

The localization of GH and its receptor (3) in the neural retina of embryonic chicks suggests GH may act as an autocrine or paracrine factor during retinal development. The extrapituitary production of GH in other target sites of GH action (54, 55) similarly supports the view that GH may act as a local growth factor during development (56-59). Roles for endogenous GH in ocular function have, however, yet to be determined.

In summary, these results demonstrate the presence of GH and GH mRNA in ocular tissues of embryonic chicks. Although GH is widespread in the neural retina of early embryos, it is particularly abundant in the RGCs. The local production of GH in the eye during chick embryogenesis suggests hitherto unsuspected roles in ocular function.

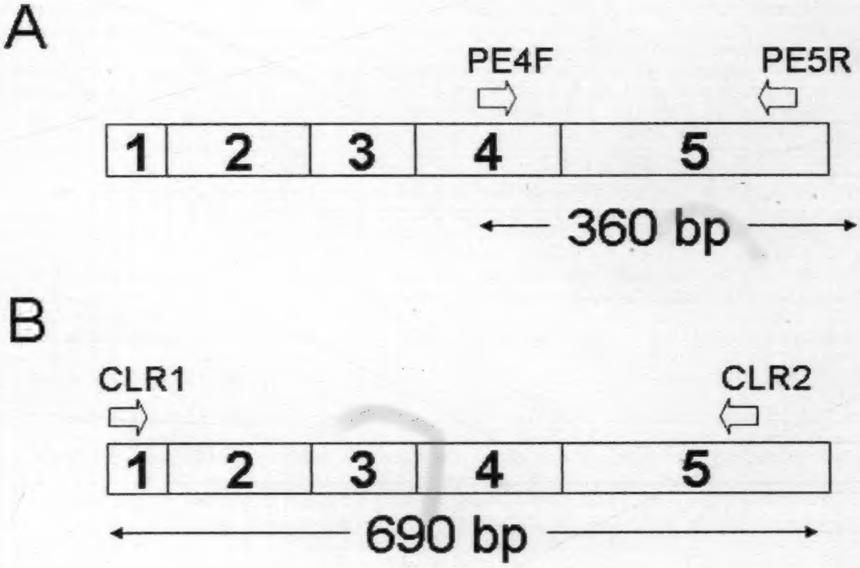
A
PE4F
PE5R
1
2
3
4
5
360 bp
B
CLR1
CLR2
1
2
3
4
5
690 bp

Fig. 2.1. Oligonucleotide primers of the chicken pituitary GH gene (7), designed to generate cDNA fragments of 360 bp (**A**) from exons 4 and 5 (primers PE4F and PE5R) (Takeuchi *et al.*, Ref. 5) and (**B**) 690 bp (primers CLR1 and CLR2, from exons 1–5) (Render *et al.*, Ref. 8).

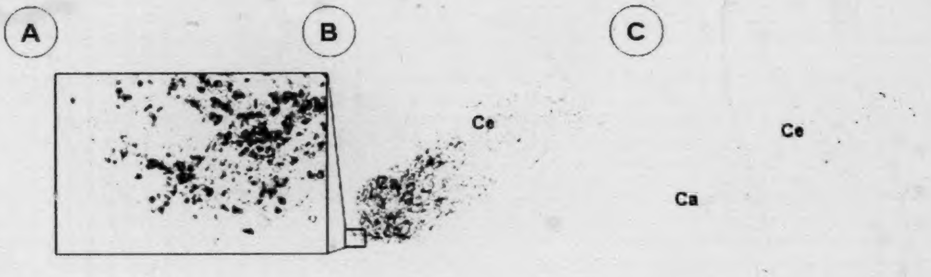

Fig. 2.2. *In situ* hybridization of GH mRNA in the pituitary glands of slaughterhouse (42-d-old) chickens. (**A**) Hybridization with a 690-bp DIG-labeled HindIII antisense probe for GH mRNA, showing cytoplasmic staining in individual somatotrophs. Magnification, X400. (**B**) Hybridization with the antisense probe, showing specific staining in the caudal (Ca) lobe of the anterior pituitary gland. Magnification, X40. (**C**) The caudal (Ca) and cephalic (Ce) lobes of the anterior pituitary gland are not labeled in the presence of the Not1 sense probe. Magnification, X40.

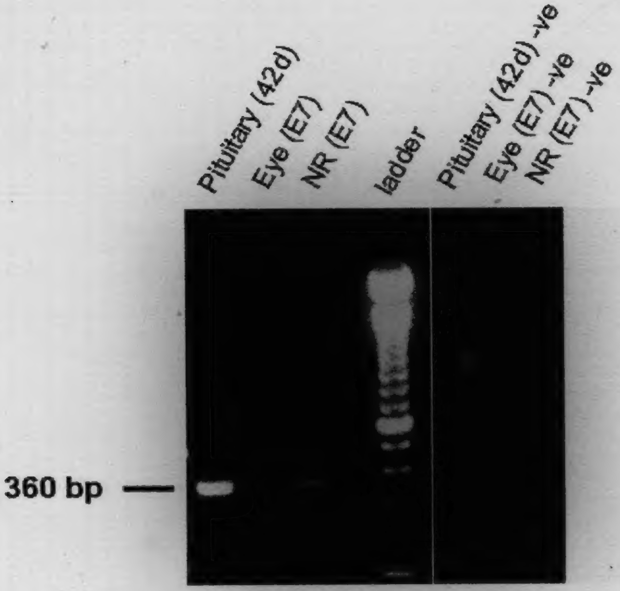
Pituitary (42d)
Eye (E7)
NR (E7)
ladder
Pituitary (42d) -ve
Eye (E7) -ve
NR (E7) -ve
360 bp

Fig. 2.3. RT-PCR of mRNA extracted from the eyes and neural retinas (NR) of chick embryos (pooled tissues from at least six embryos) at E7 in the presence of oligonucleotide primers PE4F and PE5R. RT-PCR of mRNA from the pituitary glands of slaughterhouse (42-d-old) chickens acted as a positive control. PCRs with mRNA that was not reverse transcribed with Thermoscript (-ve) served as negative controls. Faint primer-dimer bands are visible at the *bottom* of the gel. The data are representative of at least three RT-PCRs using separate tissue pools.

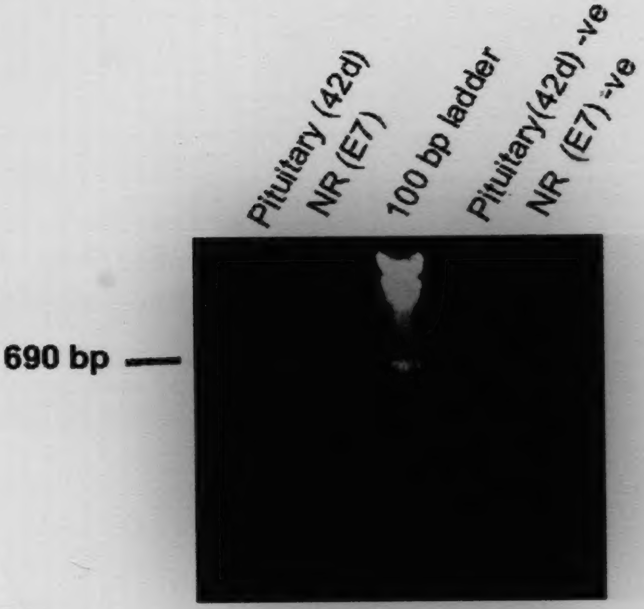
Pituitary (42d)
NR (E7)
100 bp ladder
Pituitary(42d) -ve
NR (E7) -ve
690 bp

Fig. 2.4. RT-PCR of reverse-transcribed mRNA extracted from the neural retina (NR) of chick embryos (pooled tissues from at least six embryos) at E7, in comparison with mRNA from the pituitary glands of slaughterhouse (42-d-old) chickens, using oligonucleotide primers CLR1 and CLR2 (8). Reactions with mRNA in the absence of reverse transcriptase (Thermoscript) served as negative controls (-ve). Representative data are from at least three RT-PCRs.

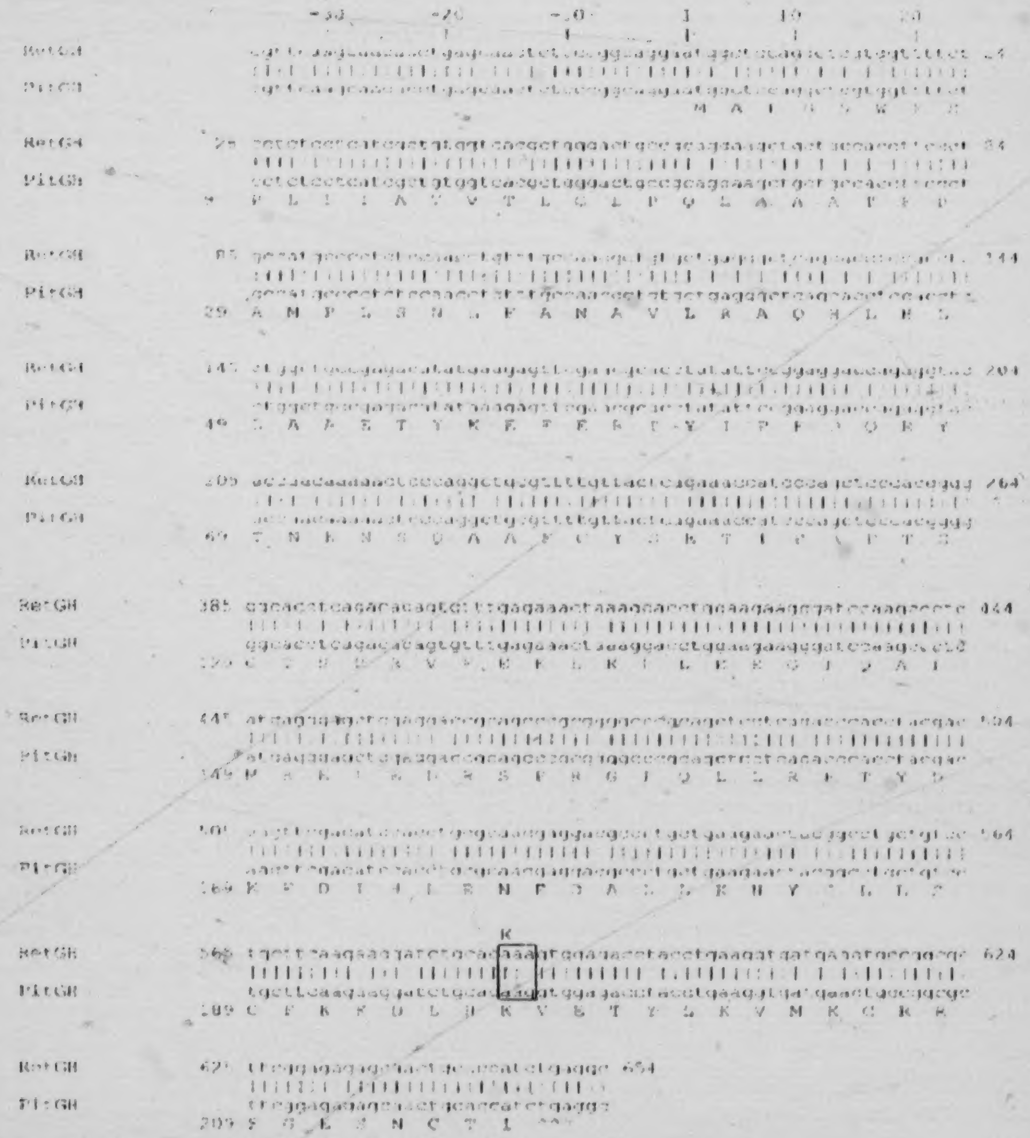

Fig. 2.5. Nucleotide and amino acid sequence of retinal GH cDNA (RetGH), in comparison with pituitary GH cDNA (PitGH) (Tanaka *et al.*, Ref. 7). The initiation site is the ATG codon for methionine. The 5' flanking untranslated region is indicated as -36 to -1. The first 25 amino acids are the signal sequence for the 216-amino-acid prohormone. The base pair substitution is indicated in the boxed codon. The GenBank accession no. for retinal GH mRNA is AY373631. The GenBank accession no. for neural retina GH protein is AAQ81586.

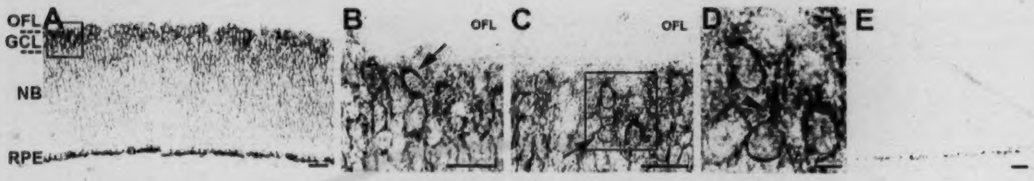

Fig. 2.6. *In situ* hybridization using a Dig-labeled riboprobe for GH mRNA. (**A**) Note the strong GH mRNA staining in the ganglion cell layer (GCL), and its absence in the OFL (NB, neuroblastic layer; RPE, retinal pigmented epithelium). (**B**) – (**D**) GH mRNA in the cytoplasm of the large, rounded cells below the optic fiber layer (OFL, arrows). Note the presence of GH mRNA in a nascent axon (arrowhead). (**E**) Absence of labeling when the antisense probe is replaced by the sense probe. Scale bars = 20 μm

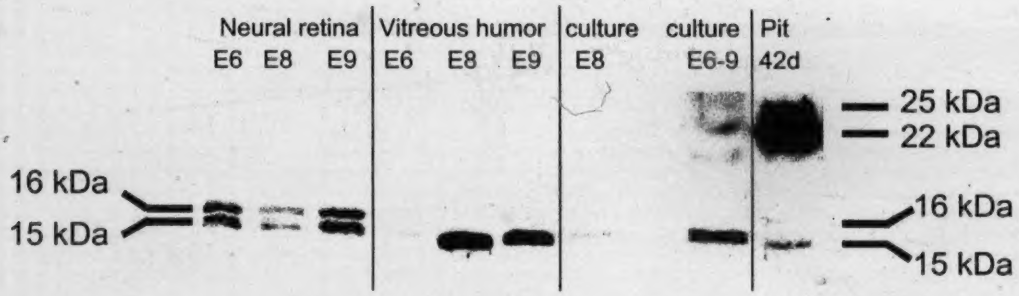
Neural retina
E6 E8 E9
Vitreous humor
E6 E8 E9
culture
E8
culture
E6-9
Pit
42d
16 kDa
15 kDa
25 kDa
22 kDa
16 kDa
15 kDa

Fig. 2.7. Western blotting of GH-immunoreactive proteins in the neural retina and vitreous humor of chick embryos (pooled tissues from at least three embryos) at E6, E8, and E9, in comparison with proteins in the pituitary (Pit) glands of slaughterhouse (42-d-old) chickens and proteins in culture media after the incubation of retinal tissues. Representative data are of at least three Western blots on separate tissue pools. The lanes were loaded with 60 μg neural retina protein, 5 μg vitreous protein, 20 μg culture media protein, and 3 μg pituitary protein.

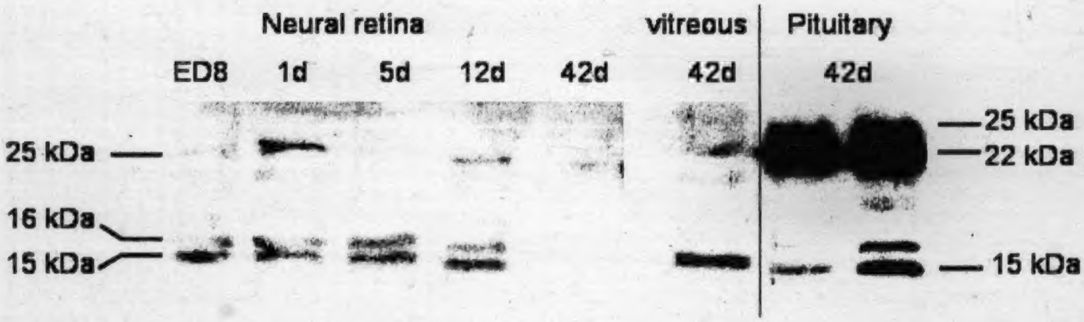
Neural retina
vitreous
Pituitary
ED8
1d
5d
12d
42d
42d
42d
25 kDa
16 kDa
15 kDa
25 kDa
22 kDa
15 kDa

Fig. 2.8. Western blotting of GH-immunoreactive proteins in the neural retina of ED8 chicks (pooled tissues from at least three birds), and in 1-, 5-, 12-, and 42-d-old chicks, in comparison with proteins in the vitreous and pituitary of slaughterhouse (42-d-old) chickens. Data are representative of at least three Western blots on separate tissue pools. The lanes were loaded with 80 μg neural retina protein, 3 μg vitreous protein, and 3 μg pituitary protein.

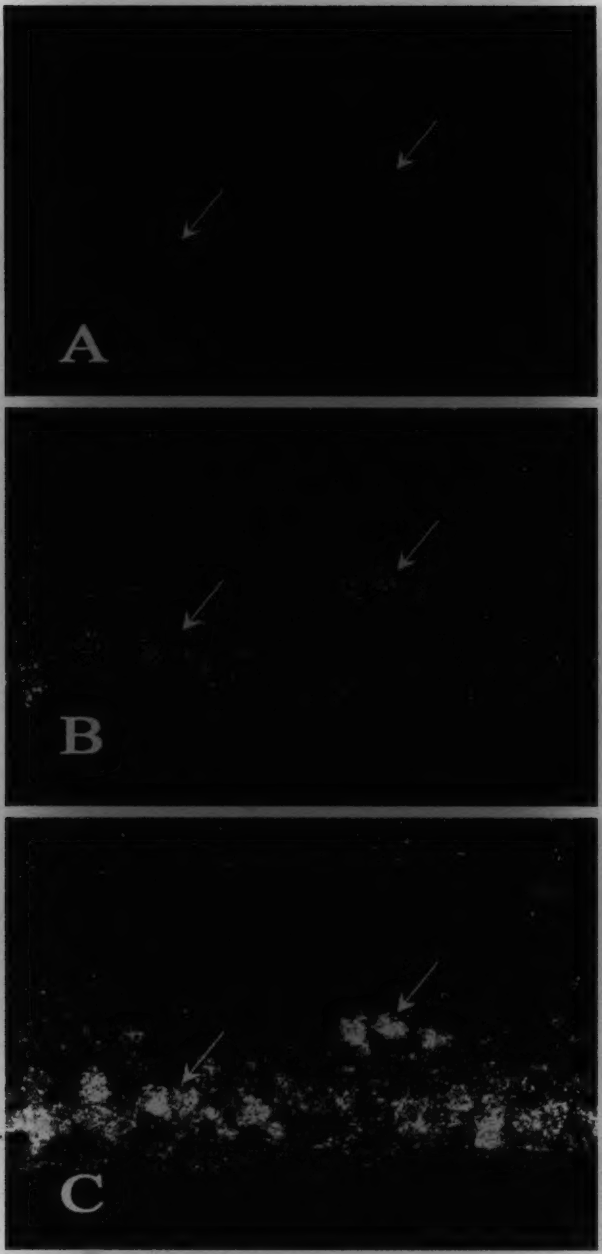

Fig. 2.9. Immunocytochemical staining in the neural retina of E8 chick embryo eyes for RGCs (39.4D5, *red*) (**A**) and GH (*green*) (**B**). Colocalization of GH in RGC-labeled cells (**C**) is indicated by the *yellow image*. *Arrows* indicate the RGCs. Magnification, X1000. Representative data are for at least six embryos.

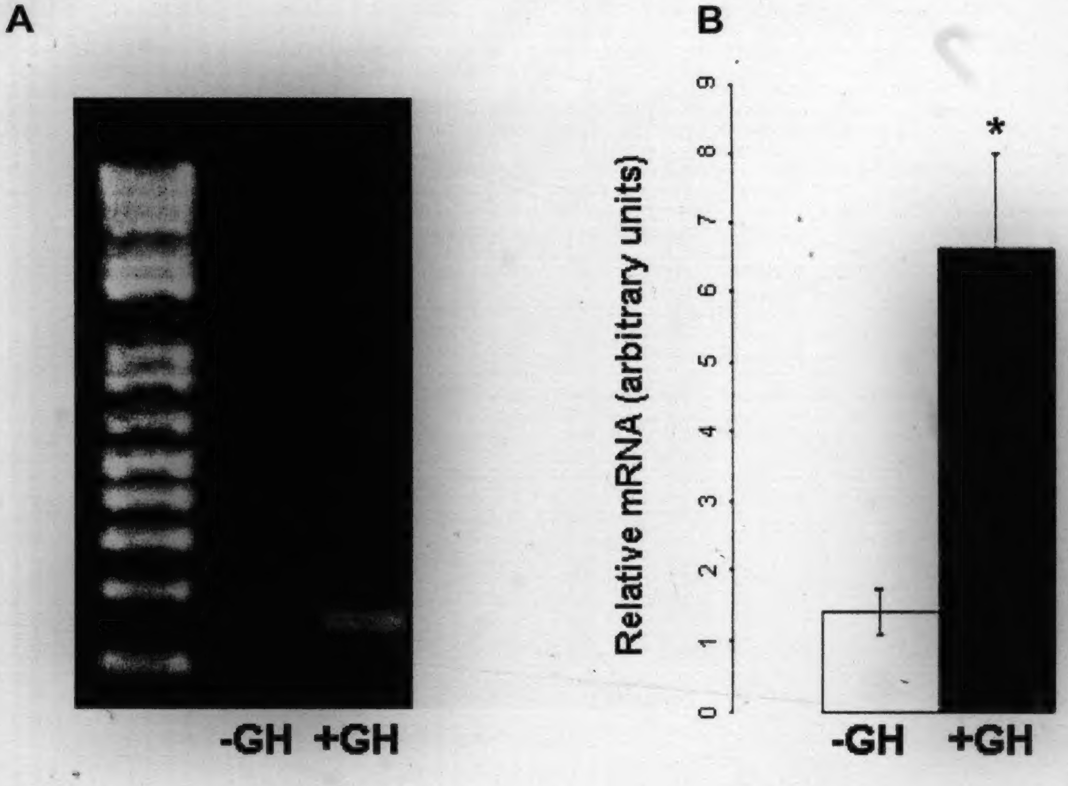

Fig. 2.10. GH action in the neural retina of E8 chick embryos after 48 h culture in the presence (+GH) or absence (-GH) of 10^{-6} M chicken GH. (**A**) RT-PCR of reverse-transcribed retinal mRNA in the presence of oligonucleotide primers designed to amplify a 147-bp fragment of chicken IGF-1 cDNA. The data are representative of six untreated neural retinas and six treated neural retinas. No cDNA fragments were generated from mRNA that had not been reverse transcribed (data not shown). (**B**) IGF-1 mRNA determined by real-time PCR in the GH-treated neural retinas (-GH; n = 6), in comparison with untreated neural retinas (+GH; n=6). The data are expressed relative to 18S rRNA in each sample (arbitrary units X 10^{-4}). Means ± SEM. The *asterisk* indicates a statistical difference (Student's *t* test), $P < 0.01$ between the groups.

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Chapter 3

Growth Hormone and its Receptor in Projection Neurons of the Chick Visual System: Retinofugal and Tectobulbar Tracts

Chapter 3: Growth Hormone (GH) and its Receptor in Projection Neurons of the Chick Visual System: Retinofugal and Tectobulbar Tracts[1]

3.1 Introduction

It is now well established that growth hormone (GH) is primarily synthesized by the anterior pituitary postnatally and acts on distant targets in an endocrine manner (Harvey et al., 1995). GH has, however, been detected in numerous extrapituitary sites including tissues of the nervous system. In the chick embryo, GH is found in the neural tube, brain vesicles, otic vesicles, semilunar, facial, acoustic and glossopharyngeal nerve ganglia, and in the trigeminal, vagal and brachial nerves (Harvey et al., 2000, 2001, Murphy and Harvey, 2001). As the central and peripheral nervous systems are established target sites of GH action, GH could thus act as a local growth factor in neural function. This possibility is supported by the presence of the GH receptor (GHR) within the same neural tissues. For instance, GHR / (binding protein) BP immunoreactivity is found in the neural tube, in developing brain vesicles, in the otic vesicles and in semilunar, facial / acoustic and glossopharyngeal nerve ganglia (Harvey et al., 2000, 2001).

GH has also recently been detected within the neural retina of early embryonic chicks, where it has been shown to prevent cell death (Harvey et al., 2000, Baudet et al., 2003, Sanders et al., 2005). GH immunoreactivity is particularly abundant in retinal ganglion cells (RGCs) of early chick embryos and likely reflects local synthesis, since GH mRNA is also detected within cells of the ganglion cell layer (GCL). The possible presence, distribution and ontogeny of GH and its receptor in other tissues of the visual system is, however, unknown and was therefore the focus of the present study.

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[1] A version of this chapter has been submitted to *Neuroscience*: Baudet ML, Rattray D, and Harvey S Growth hormone and its receptor in projection neurons of the chick visual system: retinofugal and tectobulbar tracts. Darrien Rattray assisted with the immunohistochemistry (Fig. 3.4A-D).

3.2 Materials and methods

Tissues

Fertile eggs (from the University of Alberta Poultry Unit) from White Leghorn hens were incubated at 37°C in humidified air. Whole embryos or heads were collected at embryonic day (E) 7 of the 21 day incubation period. Upon collection, they were rapidly fixed in Carnoy's reagent (Murphy et al., 2003) for immunohistochemical analysis. Tissues derived from the retinas and optic tectum were separately pooled and placed into RNA later® for subsequent RNA extraction (Ambion, Austin, Texas, USA) and RT-PCR analysis.

Immunohistochemistry

Fixation. Whole heads or embryos were fixed in freshly prepared Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) overnight at 4 °C. They were then washed (2 x 15 min) in PBS (pH 7.4) and dehydrated in a graded series of ethanol (50%, 2 x 15 min; 70%, 30 min; 95%, 30 min; 100%, 2 x 30 min) and cleared with Hemo-De (Fisher, Edmonton, Alberta, Canada) for 30 min. Tissues were then infiltrated with paraffin wax overnight at 60 °C under normal atmospheric pressure or for 3 hours under vacuum. Serial transverse (unless otherwise indicated) tissue sections (6 μm) were taken using a microtome and mounted onto charged slides (Fisher). For the retina, the sections were always taken from central areas.

Brightfield microscopy. Immunocytochemical staining was performed with commercial reagents (Vector Laboratories, Burlington, Ontario, Canada) using the ABC (Avidin:Biotinylated enzyme Complex) method (Hsu et al., 1981). Sections were cleared in Hemo-De (2 x 5 min), rehydrated in a graded series of ethanol (100%, 2 x 5 min; 95%, 2 min; 70%, 2 min; 50%, 2 min; distilled water, 2 min), equilibrated in PBS (2 min), then incubated in H_2O_2 (1% in 50% methanol) to block endogenous peroxidase. The sections were washed in PBS (3 x 5 min) and then incubated in PBS containing 10% normal goat serum (NGS, Sigma, Oakville, Ontario, Canada) and 0.4% tween for 1h. The slides were subsequently incubated overnight at 4 °C with specific antibodies for chicken GH (rabbit anti-chicken α-cGH-1; diluted 1:1,000 in 1% goat serum) (Harvey et al., 2003) or chicken GHR (mouse anti-chicken GHR; diluted 1:1,000 in 1% goat serum) (Hull et al., 1999), which was raised against a synthetic portion (CH 17) of the extracellular domain of the chicken GHR (Bingham et al., 1994). After incubation, the slides were washed (3 x 5 min) in PBS and incubated for 1h at room temperature in biotinylated goat anti-rabbit IgG (Sigma, 1:500). The slides were then washed in PBS (3 x 5 min) and incubated in ABC reagent for 1h at room temperature and washed again. Staining was visualized using the chromogenic substrate diaminobenzidine tetrahydrochloride (DAB; Sigma), which resulted in a brown coloration. Preabsorption (for 2h at room temperature) of the primary antibodies, using excess (100 μg/ μl) purified chicken GH (National Hormone and Peptide Program, Torrance, California, USA), or GHR (CH 17, generously provided by W.H. Baumbach, American Cyanamid, Princeton, New Jersey, USA) provided negative controls and completely blocked specific staining. Other controls included the omission of the secondary antibody and the replacement of the primary antibodies with PBS. Digital images were brightness, color and contrast balanced.

Confocal microscopy. The sections were similarly dehydrated, blocked with 10% NGS (in PBS containing 0.4% tween) for 1 h and incubated overnight at 4 °C with the GH or GHR antibodies (diluted 1:1,000), or with a mouse monoclonal antibody (39.4D5, Islet-1) (Austin et al., 1995, Baudet et al., 2003), which is specific for a nuclear antigen of retinal ganglion cells (RGCs) (diluted 1:1) or with a mouse monoclonal antibody (3A10) that detects a neurofilament associated antigen (diluted 1:500 from concentrate,164 μg/ml). Both monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. After incubation with the primary antibodies, the sections were incubated for 1 h at room temperature in goat anti-rabbit IgG conjugated to Alexa Fluor 488 (F(ab')$_2$ fragment, 2mg/ml, Molecular Probes), at a dilution of 1:500. The sections were then washed (3 x 10 min), incubated for an additional hour in goat anti-mouse IgG conjugated to Alexa Fluor 555 or Alexa Fluor 647 (F(ab')$_2$ fragment, Molecular Probes), at a dilution of 1:500, washed again (3 x 10 min) and mounted with Gel mount (Biomeda, Foster City, CA). In some cases, nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, Oregon, USA) for 5 min, then washed (3 x 10 min) prior to mounting. The labeled sections were examined using a LSM 510 confocal microscope (Carl Zeiss, Göttingen, Germany), equipped with appropriate lasers, using an optical slice of one airy unit. Digital images were brightness and contrast balanced.

RT-PCR

Total RNA was extracted from tissues using RNeasy Mini or Midi kits (Qiagen, Mississauga, Ontario, Canada) and in some cases, mRNA was obtained using Oligotex mRNA Mini kit (Qiagen). Total RNA (0.1 μg-5 μg) or mRNA (0.165 μg) were reverse transcribed (for 50 min at 50 °C) into cDNA using (10 U/μl) Superscript III RNAse H$^-$, 0.025 μg/ μl oligo $(dT)_{12\text{-}18}$, 5mM DTT, 1x First Strand Buffer and 1mM each dNTPs (all from Invitrogen, Burlington, Ontario, Canada). RNA templates were digested (20 min at 37 °C) using RNAse H and the newly synthesized cDNA was amplified using 0.1 U/μg Platinum *Taq* DNA Polymerase High Fidelity, 2mM $MgSO_4$, 1x High Fidelity PCR buffer, 0.2 mM each dNTPs (all from Invitrogen) in the presence of 0.2 μM each forward and reverse oligonucleotide primers designed to amplify a 1780 bp fragment spanning the extracellular, transmembrane and intracellular domains of chicken GH receptor (GHR) mRNA (Burnside et al., 1991) (forward primer: cGHR-ext-S: 5' TGGATCTTCGGCATCTGCTG 3' and reverse primer: cGHR-InA 5'TGTGCTCACATAGCCACAAG 3'). As a specific GH-response gene (GHRG-1) has been identified as a marker of GH action in chicks (Agarwal et al., 1995, Radecki et al., 1997, Harvey et al., 2002), its possible expression was also examined by RT-PCR, using oligonucleotide primers designed to amplify a cDNA fragment spanning 619 bp of the 862 bp GHRG-1 mRNAsequence (Agarwal et al., 1995) (forward primer, GHRG-1-F2: 5' GGAACTGTGTGTCTGCCTCA 3'and reverse primer, GHRG1-R2: 5'ATTCAGCACCCGATGAACTC 3'). mRNA from the liver of adult chicken was used as a positive control, since the liver is an acknowledged target site for GH action. All the primers were designed using OligoPerfect software (Invitrogen) and synthesized by Sigma Genosys (Oakville, Ontario, Canada). The initial denaturation was at 94 °C for 2 min and was followed by 38 cycles of PCR (94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 68 °C for 105 sec (GHR) or 45 sec (GHRG-1) and a final extension, for 10 min. PCR products were visualized on 0.4 μg/ml ethidium bromide gels. To assess the specificity of the signal, Superscript III RNAse H$^-$ was omitted during the RT reaction or DNA templates were not added to PCR reactions.

In situ hybridization

Cloning. To synthesize GH mRNA probes a 690 bp full-length pituitary GH cDNA obtained by using the primers CLR1 and CLR2 was subcloned into the PCR II-TOPO vector (Invitrogen), as detailed previously (Baudet et al., 2003). The sequence of the insert was determined by the University of Alberta DNA Core Facility.

Probe synthesis. The purified vectors were linearized using the restriction endonucleases NotI and HindIII for the subsequent production of sense and antisense probes, respectively. Digoxigenin (DIG)-labeled antisense and sense riboprobes were synthesized by *in vitro* transcription with T7 and SP6 RNA polymerase, respectively, and DIG RNA labeling mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Probe concentration was determined by dot blot analysis against a DIG-labeled RNA standard.

Hybridization procedure. E7 chick embryo heads were fixed overnight at 4 °C in 4% paraformaldehyde in PBS, rinsed in PBS (2 x 5 min), and cryoprotected by washes in a gradient series of sucrose (12%, 16%, 20% sucrose, 2 hours each). The heads were then embedded in O.C.T. compound (Electron Microscopy Sciences, Fort Washington, PA, USA) and frozen in liquid nitrogen. 10 μm tissue sections were cut using a cryostat, mounted onto charged slides (Fisher, Edmonton, Alberta. Canada) and stored at -80 °C until processed. Before use, the tissue sections were incubated at 60 °C for 1h, washed in diethylpyrocarbonate (DEPC)-treated water and then in DEPC-PBS. The sections were then incubated in fresh 0.1% DEPC-PBS (2 x 15 min) to remove RNase, treated with proteinase K (10 μg/ml, 10 min, 37 °C), postfixed in 4% paraformaldehyde (10 min), and incubated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. The sections were then prehybridized with 50% formamide / 2x saline sodium citrate (SSC) (30 min, 60°C) and hybridized in a moist chamber (overnight, 60 °C) with hybridization solution (50% formamide, 2x SSC, 250 μg/ml tRNA, 50 μg/ml heparin, 2% blocking solution), and denatured (5 min at 80 °C) sense or antisense DIG-labeled riboprobes at concentrations of 0.4 to 2 μg/ml. The sections were sequentially washed in 2x SSC (10 min), 2x SSC / 50% formamide (45 min, 45 °C), 2x SSC (3 x 15 min), 2x SSC (30 min, 37 °C), 0.1x SSC (60 min, 60 °C), and then washed (2 x 5 min) in buffer one (0.1 M Tris, 0.15 M NaCl, pH 7.50). Immunohistochemical detection of the hybridized probe was achieved by incubation (2h) with an anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics) in 1% blocking solution (Roche Diagnostics). The sections were then washed in buffer one (2 x 15 min), equilibrated (5 min) in buffer two (0.1 M Tris, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5), and incubated in buffer two containing a commercial substrate for alkaline phosphatase [5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium chloride; Roche Diagnostics] until color developed. The color development was stopped by washing in Tris-EDTA buffer (2 x 5 min). The sections were then rinsed in PBS (3 x 5 min) and mounted with an aqueous medium (Crystal / Mount, Biomeda, Foster City, CA, USA).

3.3 Results

GH is present and expressed in RGCs and their axons at E7

Since we previously found GH immunoreactivity in the RGCs of E7 embryos (Baudet et al., 2003), this finding was first confirmed, as a positive control. As expected, GH immunoreactivity was present in the cytoplasm (arrow, Fig. 3.1A) and in a subcompartment of the nucleus (arrowhead, Fig. 3.1A) of the large cells below the OFL. These cells were RGCs as they were immunoreactive for the islet-1 marker (arrows, Fig. 3.1B-C). No signal was detected when the primary antibodies were replaced by their respective serum (data not shown). GH immunoreactivity was also abundant in the optic fiber layer (OFL) of the E7 chick retina (Fig. 3.1A-C).

GH is located in retinofugal fibers

In chick embryos, RGCs project their axons to the optic tectum, located in the dorsal mesencephalon (LaVail and Cowan, 1971) from E6 (Mey and Thanos, 1992). GH immunoreactivity was present in RGC axons in the optic nerve head, where the axons coalesce before exiting the eye, and in the optic nerve at E5 (Fig. 3.2A) and at E7 (Fig. 3.2D-E). DAPI counterstained cells (arrowhead) within the optic nerve are shown in Fig. 3.2F. GH was also present in the optic chiasm at E7-8 (Fig. 3.2F-G) where the axons of the optic nerve decussate, and in the *stratum opticum* and *stratum griseum et fibrosum superficiale* of the optic tectum (Fig. 3.2I), where RGC axons terminate (Ramón y Cajal, 1911). Neurofilament antibody was used as a topographical marker to locate RGC axons (Fig. 3.2B, F, H, J) and it colocalized with GH in the optic nerve head and optic nerve

(Fig. 3.2C and inset), in the optic chiasm (Fig. 3.2F,H) and the *stratum opticum* of the optic tectum (Fig. 3.2K), as suggested by the orange-yellow coloration on these image overlays.

GH is located in the optic tectum at E4-8, prior to formation of the *stratum opticum*

GH was detected in the developing optic tectum at E4, before the first RGC axons reach this tissue (at E6) (Mey and Thanos, 1992). GH was present in the superficial tectobulbar tract in the marginal zone at E4 (arrows, Fig. 3.3A-B) and at E7 (Fig. 3.3E-F), where it colocalized with the neurofilament marker (arrows, Fig. 3.3C-D, G-H). GH was also ubiquitous in cells of the subjacent ventricular zone at E4 (Fig. 3.3B) and at E7 (Fig. 3.3F). As RGC axons reach the optic tectum at E6, GH immunoreactivity could be attributed, in part, to sequestration from retinofugal fibres. However, the *GH* gene was also expressed in the developing optic tectum by E7, since GH mRNA was detected, using *in situ* hybridization, in cytoplasm of cells of the ventricular zone adjacent to tectobulbar axons, which form the intermediate zone at this stage (Fig. 3.3I-M). Some of these cells were spindle shaped (arrows, Fig. 3.3L-M), as previously described (LaVail and Cowan, 1971). No signal was detected when the antisense riboprobe was replaced by the complementary sense probe (Fig. 3.3N). By E8, GH was clearly present in radially oriented fibres spanning the optic tectum (arrowhead, Fig. 3.3O) as well as in tangentially oriented tectobulbar fibres in the intermediate zone (arrow, Fig. 3.3O). Neurofilament-associated protein immunoreactivity was restricted to the radial and transverse fibers of the intermediate zone at the same stage of development (Fig. 3.3P) and appeared to be colocalized (yellow-orange) with GH immunoreactivity in this layer (arrows, Fig. 3.3Q-R). No signal was detected when the primary antibodies were replaced by their respective serum (Fig. 3.3S). The tectobulbar tract progressively sinks between E6 and E9 beneath the tectal surface following the optic tectum rostrocaudal gradient of development (Goldberg, 1974). GH (Fig. 3.3T,V) and neurofilament-associated protein colocalized (Fig. 3.3U-W) in the tectobulbar axons of the caudal marginal zone (Fig. 3.4U) and rostral intermediate zone (Fig. 3.3W) of the E8 optic tectum.

GHR protein distribution mirrors that of GH in the visual system

The possibility that extraretinal tissues of the visual system are local site of GH action was investigated by determining the distribution of GHR in the retinofugal tract and in the optic tectum. Like GH, GHR was found in the optic nerve head (Fig. 3.4A, C) and in the optic nerve (Fig. 3.4A, D), in the optic chiasm (Fig. 3.4E-G) and in the optic tract (Fig. 3.4F-G) using immunohistochemistry. No signal was detected when the primary GHR antibody was replaced by normal serum (Fig. 3.4B). In addition, GH and GHR immunoreactivities appeared similarly distributed within the optic tectum. Indeed using confocal microscopy, GH (Fig. 3.4H) and GHR (Fig. 3.4I) colocalized in fibres of the tectal plate and intermediate zone of the E8 optic tectum (Fig. 3.4J-K). No signal was detected when primary antibodies were replaced by their respective normal sera (data not shown).

Neural retina and optic tectum are sites of GH action

Using reverse transcription-polymerase chain reaction (RT-PCR), GHR cDNA was amplified from mRNA in the optic tectum and the amplified fragment was identical in size to that in the liver and the neural retina (Fig. 3.5A). This cDNA was not detected when reverse transcriptase or the DNA template was omitted. The possibility that tectal GHR are functional was also investigated by RT-PCR for the GH response gene, GHRG-1 mRNA. A PCR product was generated from RNA extracted from the E7 optic tectum, identical in size to liver and neural retina GHRG-1 cDNA. No signal was detected in the absence of reverse transcriptase or template cDNA (Fig. 3.5B).

3.4 Discussion

The results of these studies demonstrate that GH and GHR are present in extraretinal tissues of the visual system, in the optic nerve, optic chiasm, and optic tract in E7 chick embryos. They also colocalize within the intermediate zone and tectal plate of the developing optic tectum, where the presence of GHRG-1 mRNA, a GH response gene, indicates that it is likely to be an autocrine / paracrine GH target tissue. To the best of our knowledge, this is the first report of the presence of a local growth factor in two projecting tracts of the visual system, namely the retinofugal and tectobulbar tracts.

GH in visual system projecting axons

While GH immunoreactivity and GH mRNA has previously been found in the RGCs of E7 embryos (Baudet et al., 2003), the present results show, for the first time, the presence of GH immunoreactivity throughout retinofugal tract in the E7 OFL of the neural retina, in the optic nerve, optic chiasm and optic tract, as clearly indicated by the colocalization of GH with a neurofilament marker. Our earlier inability to detect GH staining in the OFL reflects the detachment of this fragile layer during the dissection of the neural retina, as we processed whole heads to prevent damage to the OFL or optic nerve in the present study but not in the earlier one. In addition, 4% PFA was used as a fixative in our earlier immunohistochemical study (Baudet et al., 2003), whereas Carnoy's fixative was used in the current investigation. Although we did detect a signal in the OFL of PFA fixed chicks, after whole head fixation (data not shown), this signal was of poor contrast contrary to the signal obtained after Carnoy's fixation. Carnoy's is much better than PFA for fixation of GH in chick neural tissues, as we've previously shown for cranial and peripheral nerves in early chick embryos (Murphy et al., 2003). It is of note that in the only other study on GH in embryonic chick eyes, Takeuchi et al. (2001) failed to detect GH-immunoreactivity in the neural retina and the OFL of (broiler) chick embryos. This is, however, likely to reflect the use of a heterologous antibody directed against rat GH, as this has poor cross-reactivity with chicken GH (Harvey et al., 1987).

While GH immunoreactivity was present in the RGC axons and soma, its mRNA was restricted to the RGC cell soma (Baudet et al., 2003). This raises the possibility that GH translation strictly occurs in the cell soma and not in axons, contrary to what has been previously reported for some other proteins (e.g. EphB2 receptor) (Piper and Holt, 2004), and that GH located in the axons of the OFL is derived, at least in part, from local RGC translation. Interestingly, GH has been found in the vitreous humor of E6-9 embryonic chicks (Baudet et al., 2003), which lies adjacent to the OFL. It is therefore possible that some axonal GH immunoreactivity could originate from its vitreal storage and transcytose within RGC axon to reach other central nervous system regions, similarly to BDNF, NT-3 and IGF-1 (von Bartheld et al., 2001). GH in the vitreous and the retina is, however, unlikely to reflect sequestration from systemic circulation, as the blood-retinal barrier is already functional by E7 (Liebner et al., 1997) and as the anterior pituitary vasculature is not complete until E20 (Sasaki et al., 2003).

In addition to the retinofugal tract, GH immunoreactivity was also found in the optic tectum, especially in tectobulbar axons from the earliest day studied (E4) until at least E8. GH was also found in the tangential tectobulbar and radial fibres, that have been shown to direct cell migration (Gray and Sanes, 1991). GH could thus also play an indirect role in this process. The presence of GH in the optic tectum is consistent with the finding of widespread GH immunoreactivity within the brains of E2-E5 chick embryos (Harvey et al., 2001). GH tectal immunoreactivity is likely to stem from locally translated GH, as GH mRNA was also detected in the cells of the optic tectum at E7. Early tectal GH is unlikely to be derived from the retina, as RGC axons do not reach the optic tectum, their main target, until E6 and retinotectal mature synapses are not present until at least E10 (Mey and Thanos, 2000). Retinal GH, conversely, is also unlikely to be sequestered from its tectal target, at least in early chick embryos before the first axon reach the tectum at E6 (Mey and Thanos, 1992).

GHR in the visual system

In addition to documenting the distribution of GH in the developing chick visual system, these results show that this is closely associated with the distribution of GHRs in the optic nerve head, optic nerve, optic chiasm and optic tract, and in the optic tectum in E7-8 embryos. These tissues are therefore not just sites of GH synthesis but they are also likely to be sites of GH action.

GH and GHR colocalize within the ganglion cells of the retina and the tectum and are particularly abundant in the projecting retinofugal and tectobulbar fiber tracts emanating from these cells. GH and / or GHR have previously been detected in other projecting axons: in the E3 chick facial / acoustic and glossopharyngeal nerves [GHR, (Harvey et al., 2001), in E7 chick facial / acoustic, trigeminal and vagal nerves GH, (Harvey et al., 2001, Murphy and Harvey, 2001)] and in the E40 bovine motoneurons and splanchnic nerve [GHR, (Kolle et al., 1998)]. This suggests a role in development / differentiation of long projecting neurons, especially as GH has established roles in neural differentiation elsewhere in the central nervous system (Sanders and Harvey, 2004, Scheepens et al., 2005, Aberg et al., 2006)

The presence of both GH and GHR protein in ganglion cell axons indicates these proteins might be transported from the retina to RGC targets and from the tectum to tectal ganglion cell targets via axonal translocation. Furthermore, because GH is produced in RGCs prior to innervation of RGC axons within the optic tectum, this transport is likely to be anterograde. Anterograde transport along RGC axons has been previously reported for FGF-2 (Ferguson et al., 1990), NT-3 (von Bartheld and Butowt, 2000) and BDNF (Spalding et al., 2002, Caleo et al., 2003) and is receptor mediated (Butowt and von Bartheld, 2001, 2005). It is therefore possible that GH binding sites in ganglion cell axons could similarly mediate GH axonal translocation.

The GHR antibody used here was raised against the extracellular domain of chicken GHR. A GH binding protein (GHBP) is present in chickens that is derived from the extracellular domain of the GHR and can be generated by proteolysis of the membrane receptor (Vleurick et al., 1999). It is therefore possible that the presence of full length GHR in RGC axons and optic tectum could result in the presence of a GHBP, not linked to signal transduction. However, the GHR antibody that we used does not detect any immunoreactive proteins in serum and is thought to specifically detect membrane-bound proteins (Hull et al., 1999). It is thus likely to indicate the presence of the GHR in ocular tissue rather than the GHBP. In addition, opticin (a proteoglycan) has been shown to bind GH in the vitreous and to colocalize with GH in RGCs in the eyes of early chick embryos (Sanders et al., 2003) and may thus act as a functional GHBP in the retina.

Putative roles of GH in RGCs axons and in the optic tectum

In addition to IGF-1, many genes have been shown to be GH-responsive (Berry et al., 1993, Laz et al., 2004, Holloway et al., 2006), but GHRG-1 is a specific marker of GH action in the chicken (Agarwal et al., 1995, Radecki et al., 1997). This serine protease has homology to Spi 2.1, an established GH-response gene in mammals, is inducible by exogenous GH and is deficient in GHR defective chickens. Its presence in the optic tectum (and the neural retina) is, therefore, indicative of the functionality of GHR in these tissues and thus of endogenous GH action. It is also of interest that GHRG-1 mRNA has previously been located in other tissues of the visual system (in the tractus isthmo-opticus) of E8 embryonic brains by *in situ* hybridization (Harvey et al., 2002). Actions of GH within these extraretinal centres have, however, yet to be determined.

Within the neural retina, GH has been shown to prevent cell death during the first wave of developmental apoptosis, which peaks at E7 (Sanders et al., 2005). The immunoneutralization of endogenous GH activates caspase-3 activity, PARP-1 cleavage and the death of RGCs (Harvey et al., 2006, Sanders et al., 2006). Cell death during the first wave of apoptosis is thought to affect neuroblasts or early postmitotic cells (Rager, 1980, Frade et al., 1997, de la Rosa and de Pablo, 2000), whereas cell death occurring during the second wave, which peaks at E12, affects mainly RGCs after they have innervated their target (Rager and Rager, 1978, Hughes and McLoon, 1979, Frade et al., 1997). The intense immunoreactivity of GH in RGC axons within the optic nerve head and the optic nerve of the retinofugal tract as early as E5, before the first axon reach the optic tectum (at E6), is thus highly unlikely to reflect retrograde transport of neuroprotective GH emanating from the tectum.

The colocalization of GH and GHR in the retinofugal and tectobulbar tract of the visual system suggests that GH might directly act on these axons possibly by promoting their outgrowth. GH involvement in neurite outgrowth has previously been suggested (Ajo et al., 2003, Ransome et al., 2004), although no direct evidence has shown to date that GH promotes neuritogenesis. For instance, it has been shown that branching of the pyramidal neurons' dendritic tree is impaired in the cortex of GHR knockout mice (Ransome et al., 2004), suggesting a role in neurite arborization, and that the growth of cellular processes is observed upon GH treatment of fetal rat cortical cell *in vitro* (Ajo et al., 2003). Moreover tubulin, an essential building block for axon outgrowth (Goldberg, 2003), is upregulated in postnatal developing rat lungs overexpressing mouse GH (Beyea et al., 2005) and polymerizes upon GH addition to the medium of CHO cells stably transfected with GHR (Goh et al., 1998).

Local GH: roles in the etiology of certain human conditions?

Congenital GH deficiency (GHD) has been associated with septo-optic dysplasia, a syndrome characterized by optic nerve hypoplasia (poorly developed optic nerve) (Nanduri and Stanhope, 1999) and with RHYNS (R*etinitis pigmentosa*, HYpopituitarism, Nephronophthisis, and Skeletal dysplasia) a syndrome characterized by impaired optic nerve head, rods and cones, retinal vasculature, and retinal pigmentation (Di Rocco et al., 1997, Hedera and Gorski, 2001). In addition, congenital GH excess has been reported in children suffering from pathologies of the optic nerve, optic chiasm and / or optic tract and, in most cases, with impaired vision (Costin et al., 1973, Crawford and Buckler, 1983, Duchowny et al., 1984, Manski et al., 1994, Drake et al., 2000). Although these syndromes are associated with pituitary / endocrine GH, the possibility that retinal and tectal / autocrine-paracrine GH acts locally either prior to pituitary differentiation or in association with endocrine GH has never been considered. This is of interest, as pituitary GH defiency and excess could also reflect such a defiency and excess in local retinal / tectal GH (if deficiency is due to a *GH* gene defect for instance). Thus, local GH might also be present and act within the human visual system, especially as GH has recently been detected within the fetal and newborn retina of rats (Harvey et al., 2006).

In summary, the distribution of GH and its receptor has been mapped, for the first time, with extraretinal tissues of the visual system during chick embryogenesis.

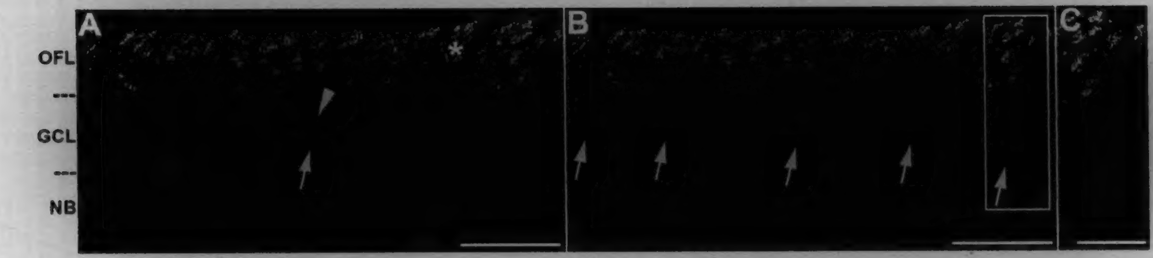

Fig. 3.1. GH immunoreactivity in the neural retina of E7 chick embryos. (**A**) GH-immunoreactivity in fascicles (asterisk) of the optic fiber layer (OFL) and in the cytoplasm (arrow) / perinuclear area of large rounded cells in the retinal ganglion cell layer (GCL). Note the subnuclear GH staining (arrowhead) in the GCL (NB, neuroblastic layer). (**B**) Image overlay of (A) and immunoreactivity for islet-1, a nuclear marker of retinal ganglion cells (RGCs). Arrows represent illustrative RGCs (in red) containing GH (in green). (**C**) Higher magnification of the boxed area in (B). Scale bars: A-B = 20 μm and C = 10 μm

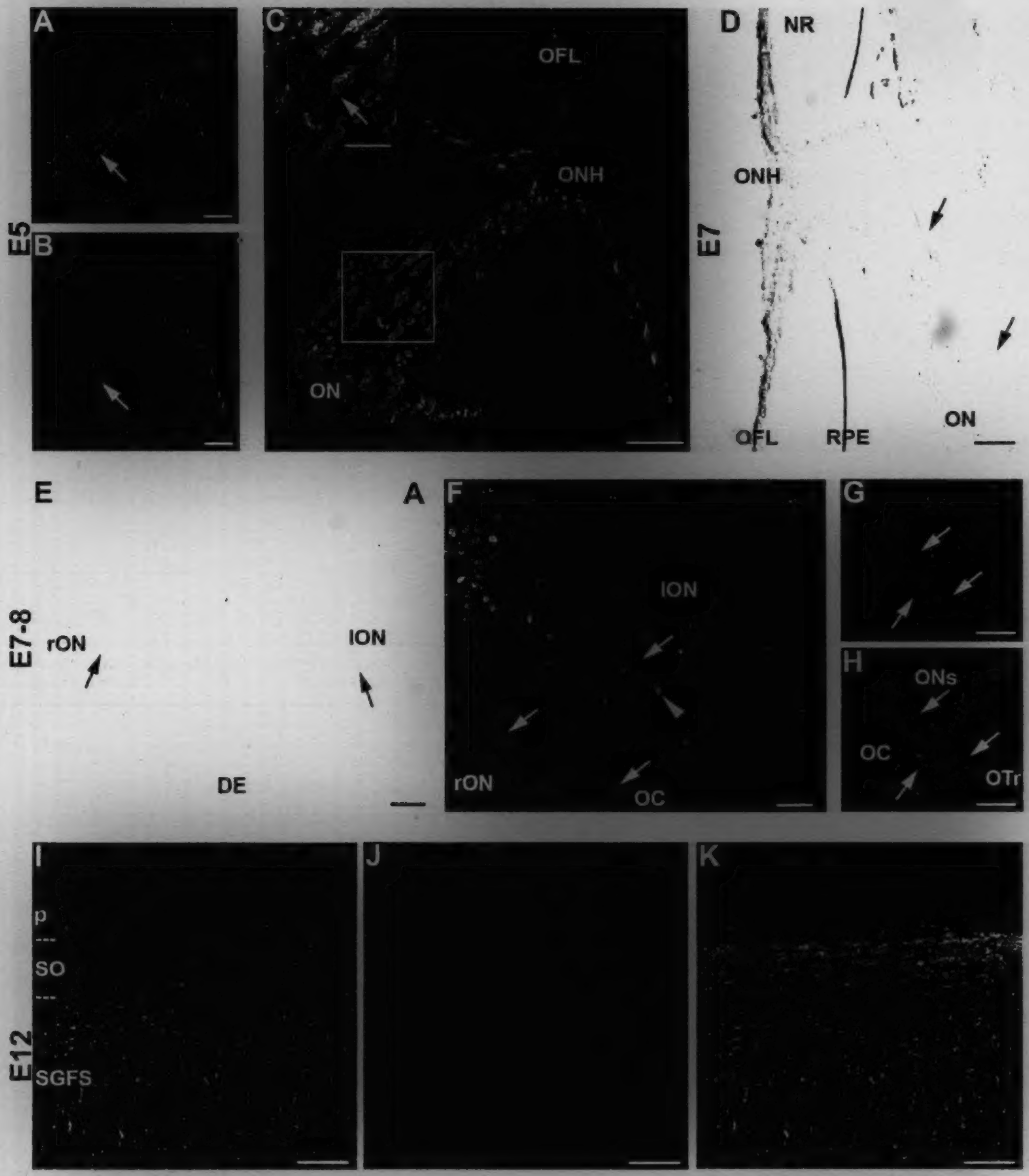
A
B
E5
C
OFL
ONH
ON
D
NR
ONH
E7
OFL
RPE
ON
E
E7-8
rON
lON
DE
A
F
lON
rON
OC
G
H
ONs
OC
OTr
I
p
SO
SGFS
E12
J
K

Fig. 3.2. GH immunoreactivity in retinal ganglion cell (RGC) axons of chick embryos. Confocal microscopy of GH immunoreactivity (green) (**A**) and neurofilament-associated protein-immunoreactivity (red) (**B**) at the back of the eye of E5 chick embryos. (**C**) Image overlay of (A) and (B) showing colocalization (yellow-orange coloration) in the optic fiber layer (OFL), optic nerve head (ONH) and optic nerve (ON). A higher magnification of GH in the fibers of the ONH is shown in the inset. The arrow points to an illustrative fascicle of the ON where GH and neurofilament-associated protein are colocalized (C, inset). (**D**) Brightfield microscopy of GH-immunoreactivity (brown) in the OFL of the neural retina (NR), ONH and ON in the eyes of E7 chick embryos. Note the presence of GH in fascicles of the ON (arrows) (RPE, retinal pigmented epithelium). (**E**) Brightfield microscopy of GH-immunoreactivity (brown) in the left (lON) and right (rON) optic nerves (arrows), just prior to decussation in the optic chiasm (OC) at E7 (DE, diencephalon). (**F**) Colocalization (yellow-orange coloration) of GH-immunoreactivity (green) in fibers immunoreactive for neurofilament-associated protein (red) in lON, rON and OC (arrows) of E7 chick embryos. Nuclei are stained in blue with DAPI. Note the presence of GH in cells of the optic nerve (arrowhead). (**G**) GH-immunoreactivity (green) in both ONs, in OC and optic tracts (OTr) (arrows). (**H**) Colocalization (yellow-orange coloration) of GH-immunoreactivity (green) and neurofilament-associated protein (red) in ONs, OC and OTr. (**I**) GH-immunoreactivity in the *stratum opticum* (SO) of the optic tectum in the brain of E12 chick embryos and in the *stratum griseum et fibrosum superficiale* (SGFS) where RGC axons terminate. (**J**) Neurofilament-associated immunoreactivity in the SO of E12 chick embryo optic tectum. (**K**) Image overlay of (I) and (J) showing colocalization (yellow-orange coloration) in the SO, but not in the SGFS. Scale bars = 20 μm except for C (inset) = 10 μm, and D, E, G and H = 50 μm

E4
A
p
MZ
VZ
B
p
MZ
VZ
C
D
E7
E
nc
F
p
MZ
IZ
VZ
G
H
p
MZ
IZ
VZ
E7
I
p
MZ
IZ
VZ
J
IZ
K
L
M
N
p
MZ/IZ
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Fig. 3.3. GH immunoreactivity in the developing optic tectum of E4-E8 chick embryos. (**A**) Brightfield microscopy of GH immunoreactivity (brown) in tectobulbar fibers of the marginal zone (MZ) of the E4 optic tectum (arrow) (VZ, ventricular zone). (**B**) Confocal microscopy of GH immunoreactivity (green) and (**C**) neurofilament-associated protein-immunoreactivity (red) in the E4 tectum. Note the presence of GH in superficial tectal fibres (arrows, B). (**D**) Image overlay of (B) and (C) showing colocalization yellow-orange coloration in the tectobulbar fibres of the MZ (arrows). (**E**) Brightfield microscopy of GH-immunoreactivity (brown) in the E7 tectum (nc, notochord). The developing tectum corresponds to the dorsal mesencephalon (between and below arrows). A higher magnification of GH in the superficial fibres of the OT is shown in the inset (arrowhead). (**F**) Confocal microscopy of GH immunoreactivity (green) in the tectobulbar fibers of the MZ and intermediate zone (IZ) as well as in cells of the VZ in E7 embryonic chicks (p, pia). (**G**) Neurofilament-associated protein immunoreactivity in the MZ and IZ. (**H**) Magnification of the image overlay of (F) and (G) showing colocalization (yellow-orange coloration) in the MZ and IZ (arrows). Nuclei are stained in blue with DAPI. (**I-J**) *In situ* hybridization using a Dig-labeled antisense riboprobe for GH mRNA in E7 chick embryos in the cytoplasm of cells located in the VZ just below the tectobulbar fibres of the MZ/IZ. (**K-M**) Higher magnification of the boxed area in (J). Note the presence of GH mRNA in the cytoplasm of spindle shaped cells (arrows). (**N**) Absence of signal when the sense riboprobe is used. (**O**) Confocal microscopy of GH immunoreactivity (green). Note the presence of GH in radial fibers (arrowhead) and in tangential fibres (arrow) of the IZ in OT of E8 chick embryos (TP, tectal plate). (**P**) Neurofilament-associated protein-immunoreactivity in the radial and tangential fibers of the IZ of the E8 OT. (**Q-R**) image overlays of (O) and (P) showing colocalization (arrows, yellow-orange colocalization) in the IZ tangential tectobulbar fibers. (**S**) Negative (normal rabbit and mouse sera) control for confocal GH and neurofilament-associated protein staining in the tectum of E8 chick embryos. (**T**) GH immunoreactivity in the fibres of the MZ of the E8 caudal tectum. (**U**) image overlay of (T) and neurofilament-associated protein immunoreactivity in tangential tectobulbar fibers of the MZ in the E8 caudal tectum. (**V**) GH immunoreactivity in the fibres of the IZ of the E8 rostral tectum, developmentally more advanced than the caudal tectum. Note the presence

of GH in the tectobulbar fibres organized in fascicles. **(W)** Image overlay of **(V)** and neurofilament-associated protein immunoreactivity in tectobulbar fibers of the IZ in the E8 rostral OT. Figures labeled according to Gray and Sanes, 1991. Scale bars = 20 μm except E = 200 μm and K-L = 10 μm

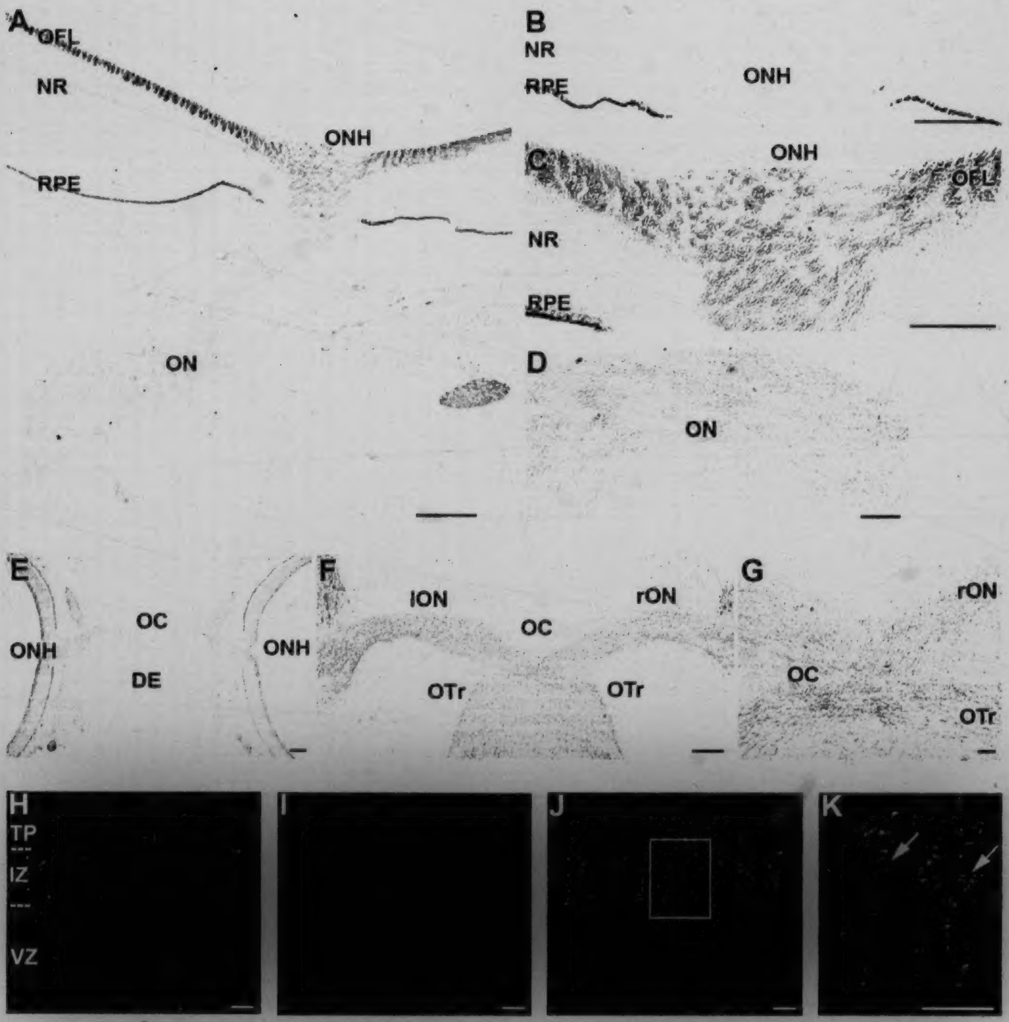
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Fig. 3.4. GH receptor (GHR) immunoreactivity and mRNA in the neural retina of E7-8 chick embryos. (**A**) Brightfield microscopy showing GHR immunoreactivity (brown) in the back of the eye of E7 embryonic chicks, in the optic fiber layer (OFL), in the optic nerve head (ONH) and in the optic nerve (ON) (NR, neural retina; RPE, retinal pigmented epithelium). (**B**) Negative (normal mouse serum) control. (**C**) Higher magnification of ONH shown in (A). (**D**) Higher magnification of ON shown in A. (**E**) brightfield microscopy of GHR immunoreactivity (brown) in a cross section of E7 chick embryo head. Note the strong immunoreactivity in both optic nerves emanating from the right and left retina, the optic chiasm (OC) and optic tract located frontally to the diencephalon (DE). (**F**) Higher magnification of left (lON) and right (rON) optic nerves and OC in (D) (OTr, optic tract). (**G**) Higher magnification of OC in (E). Confocal microscopy showing GH (green) (**H**) and GHR (red) (**I**) immunoreactivities in the tectal plate (TP) and intermediate zone (IZ) of the optic tectum of E8 chick embryos. (**J**) Image overlay of (H) and (I) showing colocalization (yellow-orange coloration) of GH and GHR immureactivities. (**K**) Higher magnification of the boxed area in (J) showing colocalized GH and GHR in tectobulbar fibers of the IZ of the E8 optic tectum. Scale bars = 20 μm except A, B E, F = 100 μm, C = 50 μm and K= 10 μm

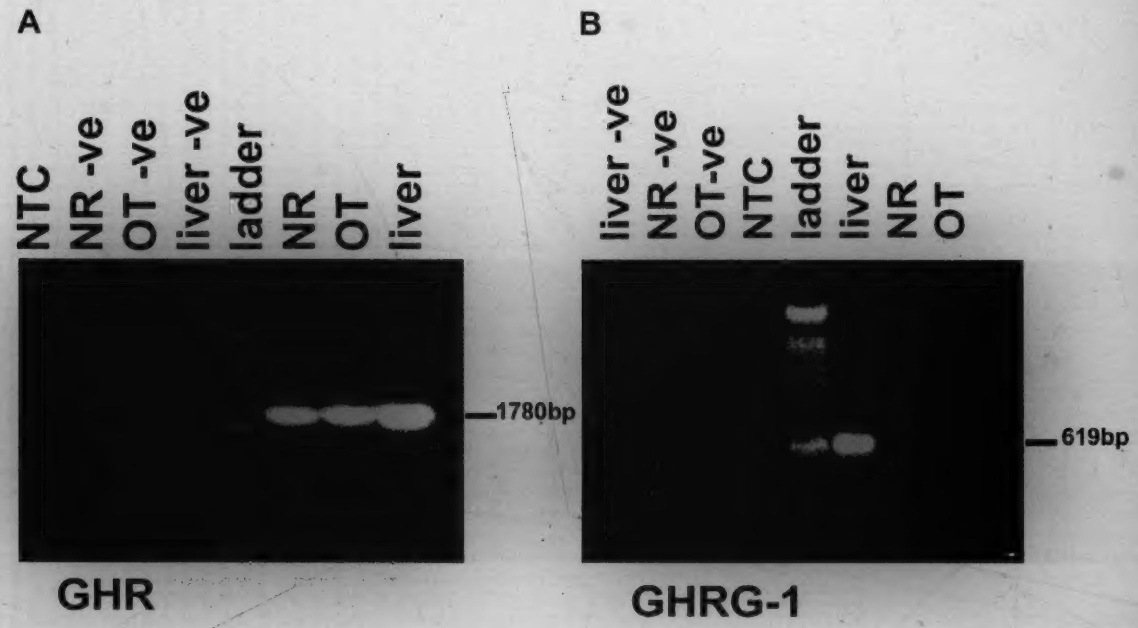
A
NTC
NR -ve
OT -ve
liver -ve
ladder
NR
OT
liver
1780bp
GHR
B
liver -ve
NR -ve
OT-ve
NTC
ladder
liver
NR
OT
619bp
GHRG-1

Fig. 3.5. RT-PCR of growth hormone receptor (GHR) (**A**) and growth hormone response gene (GHRG) -1 (**B**) mRNA in the E7-8 neural retina (NR) and optic tectum (OT), and adult liver of chickens. Abbreviations: -ve, negative; bp, base pair; NTC, non-template control; ladder (A), 1kbp plus ladder; ladder (B), 100bp ladder.

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Chapter 4

Growth Hormone Promotes Axon Growth in the Developing Nervous System

Chapter 4: Growth Hormone Promotes Axon Growth in the Developing Nervous System[1]

4.1 Introduction

Postnatally, growth hormone (GH) is primarily produced by pituitary somatotrophs and acts as an endocrine at distant target sites (1). A growing body of evidence suggests, however, that GH is also widely distributed within developing nervous tissues pre- and perinatally. Neural GH has thus been detected in early chick embryos in brain vesicles, in the spinal cord, in nerve ganglia, in peripheral nerves, and in the neural retina (2-4). GH has also been found in human fetal brain (5) and in fetal and perinatal rodent brain, [in the cortex, thalamus, hypothalamus, hippocampus, and amygdala (6, 7)] and in the rodent neural retina (8). Developing nervous tissues are also likely to be sites of GH action. Like GH, GH receptor (GHR) immunoreactivity has been detected in developing brain vesicles, in nerve ganglia, and in the neural retina of early chick embryos (2, 4, 9). GHR is similarly present in brain vesicles, the spinal cord, nerve ganglia, and neural retina of rodents (10, 11), in the developing brains of rabbits and humans (11, 12) and in the spinal cord and associated nerves of fetal cattle (13).

These studies clearly demonstrate that GH and GHR are widely distributed in the nervous system during development, but roles for GH in neurogenesis are still largely unknown. It has, however, been suggested that GH promotes cell proliferation, cell differentiation and cell survival in the development of the central nervous system (CNS) (7, 14, 15). For instance, GH promotes the proliferation of cells of neural and astrocytic lineage from fetal rat cerebral cortex *in vitro* (14) and induces cortical neuron differentiation during later development (14). Moreover, GH promotes the differentiation of astrocytes from cultures of murine stem cells whilst preventing the differentiation of cortical neurons (7, 16). Finally, GH promotes cell survival in early embryonic chick retinas *in vivo* and *in vitro* (15). In particular, GH prevents retinal ganglion cell (RGC) death, via caspase dependent and independent mechanisms (17, 18).

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[1] A version of this chapter will be submitted to *Endocrinology* for publication: Baudet ML, Rattray D, Martin BT and Harvey S Growth hormone promotes axon growth in the developing nervous system . D. Rattray (Fig. 4.2A-F) and B.T. Martin (Fig. 4.1O,P) assisted with the immunohistochemistry and *in situ* hybridization.

These results, therefore, suggest that GHRs are functionally expressed in the RGCs of early chick embryos. However, while GHR mRNA has been identified in extracts from the neural retinas of embryonic day (E) 7 chicks (9), the cellular localization of GHR mRNA is unknown and the translation of this transcript has yet to be shown. The cellular distribution of GH and of GHR mRNA and protein in the neural retina has, therefore, been determined during chick embryogenesis and correlated with the ontogenic growth of RGC axons.

The results of these studies suggest endogenous GH is involved in axon growth, as demonstrated for exogenous GH in culture of immunopanned RGCs. This hitherto unsuspected neurotropic role of GH may have clinical relevance since congenital GH deficiency has previously been associated with clinical syndromes of optic nerve hypoplasia.

4.2 Materials and Methods

Tissues

Fertile eggs (from the University of Alberta Poultry Unit) from White Leghorn hens were incubated at 37°C in humidified air. Whole embryo or whole heads were collected between E4 to 18 of the 21 day incubation period. The collection of tissues from decapitated embryos (>E7) was with approval of the University of Alberta's Animal Welfare Committee.

***In situ* hybridization**

Cloning. To synthesize GHR mRNA probes, a fragment was first amplified from liver GHR cDNA using RT-PCR [denaturation at 94 °C for 2 min followed by 38 cycles of denaturation (94 °C for 30 sec), annealing (60 °C for 30 sec), extension (68 °C for 1 min), with a final extension of 10 min], and a set of primers (forward: cGHR-Ex-S 5' ATGGATCTTCGGCATCTGCTG 3'and reverse: cGHR-Ex-A 5' GTACGTTGTCTTGATCGGAC 3') designed to amplify a 610 bp fragment encoding the extracellular domain of the chicken GHR-mRNA (Burnside et al., 1991). This PCR fragment was then electrophoresed in ethidium bromide-stained agarose gels (1.5%

wt/vol) and gel purified using a QIAquick Gel extraction kit (Qiagen, Burlington, Ontario). The pure cDNA was then subcloned into a PCR II-TOPO vector (Invitrogen, Burlington, Ontario), transformed into chemically competent TOP10 *Escherichia coli* (Invitrogen) and the cells allowed to grow overnight at 37°C. The vectors were then collected using a HiSpeed Plasmid Midikit (Qiagen) and the insert obtained by *EcoRI* endonuclease digestion and visualized on ethidium bromide-stained agarose gels. The sequence of the insert was verified by the University of Alberta DNA Core Facility.

Probe synthesis. The purified vectors were linearized using the restriction endonucleases NotI and SacI (GHR) for the subsequent production of sense and antisense probes, respectively. Digoxigenin (DIG)-labeled antisense and sense riboprobes were synthesized by *in vitro* transcription with T7 and SP6 RNA polymerase, respectively, and DIG RNA labeling mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Probe concentration was determined by dot blot analysis against a DIG-labeled RNA standard.

Hybridization procedure. E7 chick embryo heads were fixed overnight at 4 °C in 4% paraformaldehyde (PFA) in PBS, rinsed in PBS (2 x 5 min), and cryoprotected by washes in a gradient series of sucrose (12%, 16%, 20% sucrose, 2 hours each). The heads were then embedded in O.C.T. compound (Electron Microscopy Sciences, Fort Washington, PA) then frozen in liquid nitrogen. 10 μm tissue sections were cut using a cryostat, mounted onto charged slides (Fisher Scientific, Edmonton, Alberta) and stored at -80 °C until processed. Before use, the tissue sections were incubated at 60 °C for 1h, washed in diethylpyrocarbonate (DEPC)-treated water and then in DEPC-PBS. The sections were then incubated in fresh 0.1% DEPC-PBS (2 x 15 min) to remove RNase, treated with proteinase K (10 μg/ml, 10 min, 37 °C), postfixed in 4% paraformaldehyde (10 min), and incubated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. The sections were then prehybridized with 50% formamide / 2x saline sodium citrate (SSC) (30 min, 60°C) and hybridized in a moist chamber (overnight, 60 °C) with hybridization solution (50% formamide, 2x SSC, 250 μg/ml tRNA, 50 μg/ml heparin, 2% blocking solution), and denatured (5 min at 80 °C) sense or antisense DIG-labeled riboprobes at concentrations of 0.4 to 2 μg/ml. The sections were sequentially washed in 2x SSC (10 min), 2x SSC / 50% formamide (45 min, 45 °C), 2x SSC (3 x 15 min), 2x SSC (30 min, 37 °C), 0.1x SSC (60 min, 60 °C), and then washed (2 x 5 min) in buffer one (0.1 M Tris, 0.15 M NaCl, pH 7.50). Immunohistochemical detection of the hybridized probe was achieved by incubation (2h) with an anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics) in 1% blocking solution (Roche Diagnostics). The sections were then washed in buffer one (2 x 15 min), equilibrated (5 min) in buffer two (0.1 M Tris, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5), and incubated in buffer two containing a commercial substrate for alkaline phosphatase [5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium chloride; Roche Diagnostics] until color developed. The color development was stopped by washing in Tris-EDTA buffer (2 x 5 min). The sections were then rinsed in PBS (3 x 5 min) and mounted with an aqueous medium (Crystal/Mount, Biomeda, Foster City, CA).

Immunohistochemistry

Fixation. Whole heads or embryos were fixed in freshly prepared Carnoy's (60% ethanol, 30% chloroform, 10% glacial acetic acid) overnight at 4 °C. They were then washed (2 x 15 min) in PBS (pH 7.4), dehydrated in a graded series of ethanol (50%, 2 x 15 min; 70%, 30 min; 95%, 30 min; 100%, 2 x 30 min) and cleared with Hemo-De (Fisher Scientific) for 30 min. Tissues were then infiltrated with paraffin wax overnight at 60 °C under normal atmospheric pressure or for 3 hours under vacuum. Serial transverse (unless otherwise indicated) tissue sections (6 μm) were taken using a microtome and mounted onto charged slides (Fisher Scientific). For the retina, the sections were always taken from central areas.

Confocal microscopy. The sections were similarly dehydrated, blocked with 10% NGS (in PBS containing 0.4% tween) for 1 h and incubated overnight at 4 °C with the GH or GHR antibodies (diluted 1:1,000), or with a mouse monoclonal antibody (3A10) that detects a neurofilament associated antigen (diluted 1:500 from concentrate,164 μg/ml). Normal mouse (NMS) and rabbit (NRS) sera at similar dilutions were used as negative controls. 3A10 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. After incubation with the primary antibodies, the sections were incubated for 1 h at room temperature in goat anti-rabbit IgG conjugated to Alexa Fluor 488 ($F(ab')_2$ fragment, 2mg/ml, Molecular Probes), at a dilution of 1:500. The sections were then washed (3 x 10 min), incubated for an additional hour in goat anti-mouse IgG conjugated to Alexa Fluor 555 or Alexa Fluor 647 (F(ab')$_2$ fragment, Molecular Probes), at a dilution of 1:500, washed again (3 x 10 min) and mounted with Gel mount (Biomeda, Foster City, CA). In some cases, nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, Oregon) for 5 min, then washed (3 x 10 min) prior to mounting. The labeled sections were examined using a LSM 510 confocal microscope (Carl Zeiss, Göttingen, Germany), equipped with appropriate lasers, using an optical slice of one airy unit. Digital images were brightness and contrast balanced.

RGC immunopanning

Retinal ganglion cells were enriched from a retinal cell suspension using immunopanning, as previously described (15) with minor modifications. This method yields > 90% cell purity (15).

Preparation of Poly-D-lysine / laminin coated coverslips. Glass coverslips were coated, as detailed elsewhere (19-21), with 10 μg/ml poly-D-lysine (Sigma, Oakville, Ontario) for 30 min at room temperature, rinsed twice with water and dried. 50 μg/ml in EBSS (Earle's balanced salt solution, Sigma,), laminin (Sigma) was subsequently added and incubated at 37°C until ready to use. Laminin is a physiological axon growth promoting substrate for RGCs (22).

Preparation of Thy-1 dishes. Glass coverslips were first coated overnight at 4°C with 50 μg/ml goat anti-mouse IgG (Sigma) in 50 mM Tris-HCl buffer (pH 9.5), washed three times with 0.25% BSA then incubated with 55 μg/ml anti-chicken Thy-1 antibody (kindly donated by Dr. P.L Jeffrey, University of Sydney, Australia) in PBS for 2hrs at room temperature. The coverslips were washed three times with PBS, blocked with 0.25% BSA (1hr, RT) and washed once with PBS then HBSS (Hank's balanced salt solution, Invitrogen) just prior to the addition of freshly dispersed retinal cells.

Purification of retinal ganglion cells. Four neural retinas (one per E7 chick embryo) were dissected in HBSS containing 50 μg/ml gentamycin (Invitrogen) and 500 U/ml polymyxin (Sigma) and briefly centrifuged. The medium was replaced by 0.1% trypsin in HBSS (Sigma) and incubated at 37°C for 10 min. 10% (v/v) FBS (Invitrogen) and 1% (v/v) of BSA were added to inactivate the trypsin and the retinas were triturated 10 times. 10 U of DNase (Sigma) were added and the retinas were again triturated 10 times.

Dispersed retinal cells were washed twice by centrifugation (500 x g, 5 min each), resuspended in HBSS containing 10% heat-inactivated FBS (Invitrogen) and antibiotics and added to the freshly coated Thy-1 dishes. After a 1h15 incubation at room temperature, the coverslips were washed with HBSS containing antibiotics to remove any non-adherent (non-RGCs) cells and subsequently incubated with 0.125% trypsin (Invitrogen) in HBSS at 37°C for 5 min. Trypsin was inactivated by adding a HBSS solution containing 30% FBS and the cells were gently detached by squirting medium onto the coverslips. The cells were washed twice by centrifugation (2000 x g, 5 min each) and resuspended with neural basal medium (Invitrogen) containing B27 serum-free supplement (Invitrogen), 2 mM glutaMAX-I (Invitrogen) and 50 U/ml penicillin-G and 50 U/ml streptomycin-sulfate (Invitrogen) and treatment when applicable. Cells were then seeded onto poly-D-lysine / laminin coated coverslips at 200 μl per coverslip and incubated at 37°C.

Treatment of RGCs. RGCs were incubated for 3 days at 37 °C with 10^{-6}M or 10^{-9}M recombinant chicken (rc) GH (Protein laboratory Rehovot, Israel), or, as a positive control, with 50 ng/ml human BDNF (Sigma) in the presence of 10μM forskolin (Sigma) (21). A similar approach has been used to assess GH actions in mouse cortical stem cells (23) and to determine neurotropic action of other growth factors in retinal cells (21).

DiI staining. For visualization, RGCs were stained with DiI Vybrant Cell-Labeling Solutions (Invitrogen) following manufacturer's instructions. RGCs were incubated at 37°C for 20 min with 1:200 DiI solution in culture medium after three days in culture. The cells were gently washed three times with warm culture medium (10 min each) and RGC neurites were visualized with a fluorescent microscope (Zeiss) equipped with appropriate optical filters.

Axon measurements and Statistical Analysis. Axon length was measured using a phase contrast microscope equipped with an ocular eye piece. The longest neurite was measured from the edge of the cell soma to the tip of the growth cone and considered to be the RGC axon. When the cell projected more than one long neurite, the longest one was measured. Conversely, neurites smaller than one cell body (~10 μm) were not counted as they could not be distinguished from putative dendrites. A similar approach has been previously used (20, 21). For each experiment, each treatment was performed 3 times (GH) or 4 times (BDNF-forskolin) on pooled RGCs and up to 30 axons were counted per dish. Data were plotted and analyzed using Prism 4.0. Statistical differences were determined by unpaired *t*-test or one-way analysis of variance, where appropriate.

Immunocytochemistry

RGCs were fixed with 4% PFA in PBS for 15 min at room temperature. The cells were rinsed 3X with PBS and incubated with 50 mM NH_4Cl to minimize autofluorescence. The cells were then permeabilized with 0.1% Triton X-100 (Sigma) for 5 min and washed three times. The cells were blocked by incubation with 10% goat serum for 1hr at room temperature and subsequently incubated overnight at 4°C with rabbit anti-chicken αcGH-1 (24) in 1% NGS . The cells were rinsed three times in PBS (10 min each), incubated with 4 μg/ml AlexaFluor 555 goat anti-rabbit IgG (H+L, $F(ab')_2$ fragment, Molecular probes) for 1 hr at room temperature in the dark, washed again three times with PBS (10 min each, in the dark) and mounted with Prolong Gold anti-fade reagent with DAPI (Invitrogen). The primary antibody was replaced by normal rabbit serum (NRS) at the same dilution for control. The labeled cell culture were examined using a LSM 510 confocal microscope (Zeiss), equipped with appropriate lasers. To compare fluorescence levels between labeled coverslips, digital photographs were taken with identical settings and without saturation.

4.3 Results

GH is located in RGC and their axons in the retina from E4 to E14

Although GH is not present in the chick pituitary gland until E12 (25) and in the systemic plasma until E17 (26), GH immunoreactivity appeared in the developing retina as early as E4 (Fig. 4.1A-B). This immunoreactivity was present in the cytoplasm / perinuclear compartment and nucleus (arrowheads, Fig. 4.1B,E) of cells in the neuroblastic layer and in the optic fiber layer (OFL) (arrows, Fig. 4.1B,E). In the OFL, GH colocalized with the neurofilament marker (3A10), as suggested by a yellow-orange coloration (arrows, Fig. 4.1G-H) from the merged images of GH (Fig. 4.1E) and neurofilament (Fig. 4.1F) immunoreactivity. GH was similarly present in the OFL, organized in fascicles, at E8 (Fig. 4.1I), and colocalized with the same neurofilament marker (Fig. 4.1J) in this layer (yellow coloration, Fig. 4.1K-L). GH was also present in the OFL at E10 (Fig. 4.1M), at E12 (Fig. 4.1N) and somewhat at E14 (Fig. 4.1O). However no signal was detected at E18 (Fig. 4.1P) suggesting a downregulation of GH expression starting after E12. GH signal could also be detected in the soma of cells of the ganglion cell layer (GCL) at E4-18 (arrowhead, Fig. 4.1E,I,M-P), and it was particularly strong at E18. Additionally, GH immunoreactivity appeared in the inner plexiform layer at E12 (arrow, Fig. 4.1N), and was also visible at E14 (arrow, Fig. 4.1O) and E18 (arrow, Fig. 4.1P). GH was clearly present in cells of the inner nuclear layer at E18 (Fig. 4.1P) and was occasionally seen in this layer before this stage (arrow, Fig. 4.1M-O). No signal was detected when primary antibodies were replaced by their respective normal serum (Fig. 4.1C for E4 and Fig. 4.1D for E18).

GHR mRNA and protein distribution mirrors that of GH in the OFL

The possibility that RGCs are site of local GH action was investigated by using *in situ* hybridization to determine the distribution of GHR mRNA. Like GH mRNA (24), the GHR transcript was found throughout the neural retina at E7 (Fig. 4.2A) and was more abundant in the superficial layer of the neural retina, where post-mitotic RGCs are located (arrowheads, Fig. 4.2B). No signal was detected in the OFL (Fig. 4.2A,B), nor in the neural retina when the sense riboprobe was used (Fig. 4.2C). The distribution of the GHR protein mirrored that of the GHR mRNA. Using immunohistochemistry, GHR immunoreactivity was detected in the neural retina in the OFL (arrows, Fig. 4.2D-E), in the cytoplasm of large rounded cells with the morphology and location of RGCs (arrowheads, Fig. 4.2E). No signal was detected when the primary GHR antibody was replaced by normal serum (Fig. 4.2F). Thus, GH and GHR immunoreactivities appeared similarly distributed. Indeed, using confocal microscopy, GH (Fig. 4.2G) and GHR (Fig. 4.2H) colocalized in the OFL of the E7 neural retina (Fig. 4.2I). No signal was detected when primary antibodies were replaced by their respective normal serum (data not shown). Thus, E7 RGCs are likely to be sites of GH action and GH production.

Axon growth in immunopanned RGC culture

The ontogenic distribution of GH mirrors the ontogeny of RGC axon elongation (27). The possibility that GH promotes RGC axon growth was thus investigated using ganglion cell culture. RGCs were enriched from E7 chick embryo retinal cell cultures by immunopanning and found to be immunoreactive for GH (Fig. 4.3A). The signal was particularly strong in the nucleus and cytoplasm of these cells and was also present in their neurites (Fig. 4.3B,C). In contrast, no immunoreactivity was detected when the GH antibody was replaced by NRS (Fig. 4.3D).

The ability of RGCs to produce neurite outgrowth in culture in response to treatment was first assessed using 50 ng/ml BDNF and 10 μM forskolin, as a positive control, since this has previously been shown to promote axon growth of neonatal rat immunopanned RGCs in a synergistic manner (21). In response to this treatment, a significant (260%, $p<0.05$) increase in mean axon length was obtained in comparison with the PBS control in which only modest axon outgrowth was seen (Fig. 4.4A,B). The proportion of long axons was also increased upon treatment, since 44% of BDNF and forskolin treated axons were longer than 100 μm, compared to 9% in the PBS controls (Fig. 4.4C). In comparison with control RGC axons, axon length was also significantly increased upon addition of 10^{-9}M rcGH (recombinant chicken GH) ($P<0.05$) and 10^{-6}M rcGH ($P<0.001$) by 135% and 230%, respectively (Fig. 4.5A,B). The length of the axons induced by 10^{-9}M rcGH was significantly less ($P<0.001$) than that induced in 10^{-6}M rcGH (Fig. 4.5A). GH thus stimulated axon growth and higher doses of GH were more potent than lower doses. Both 10^{-9}M and 10^{-6}M rcGH promoted the extension of long axons, as compared to PBS control, since 23% (10^{-6}M) and 10% (10^{-9}M) of RGC axons were longer than 100 μm compared to 2% of the PBS controls (Fig. 4.5C).

4.4 Discussion

These results clearly demonstrate that GH is present in RGCs throughout their ontogeny, but GH is only abundant in RGC axons during a specific window of development during which axon elongation occurs. GHR mRNA was also present in cells of the GCL and GHR colocalized with GH within the OFL, suggesting it is a paracrine / autocrine site of GH action. Indeed, actions of exogenous GH on axon growth were demonstrated for the first time.

GH in RGCs and their axons

While GH immunoreactivity and GH mRNA have previously been found in the RGCs of E7 embryos (24), the present study describes, for the first time, the ontogenic appearance and extinction of GH immunoreactivity in RGC axons between E4 and E18. This temporal pattern might reflect roles of GH during neurogenesis.

GH has been shown to prevent cell death during the first wave of apoptosis (15), which peaks at E6 (28). In addition, as GH is still present in RGC soma, during the second wave of apoptosis, which occurs between E11 and E16 (27), it may similarly be involved in RGC survival during this stage. The presence of GH in RGCs and their axons by E4, before the first wave of cell death, may indicate that GH has additional roles in the developing RGCs. RGCs are the first retinal cell type to leave the cell cycle and start differentiating, beginning at E3 in the central retina (29) and this process is mostly complete by E12 (27). GH might therefore additionally influence the differentiation of RGCs (and other retinal cells) and their neuritogenesis, especially as GH has established roles in neural differentiation elsewhere in the CNS (14, 30).

It is of particular interest that GH was particularly abundant in the OFL from E4, soon after RGC axons start to form (31), until E12, after all optic fibers have spread across the optic tectum(at about E12), RGC axons' main target (32), and have innervated the tectum within the *stratum griseum et fibrosum superficiale* (at E14) (33). GH abundance in the OFL had, however, decreased after E12 and vanished by E18. The chronotopy of GH distribution in the OFL therefore strongly suggests it may participate in RGC axon outgrowth. The loss of GH from the OFL of the neural retina towards hatch is similar to the neonatal loss of immunoreactive GH in retinal extracts, determined by Western blotting (24). This suggests the actions of GH in the retina might be mostly associated with retinal development and differentiation.

GH promotes RGC axon growth *in vitro*

We show here, for the first time, that recombinant chicken GH significantly induces RGC axon growth *in vitro* in a dose dependant manner after three days in culture. GH induced a significant increase in axon length at 10^{-9}M (22 ng/ml) and at 10^{-6}M (22 μg/ml), by 135% and by 230%, respectively, compared to PBS control. These doses are within the physiological range, since GH concentrations found in the plasma of perinatal and neonatal chicks are approximately 10^{-9}M (9) and as GH affects RGC survival at 10^{-6}M in early chick embryos (15, 18). This 22 kDa rcGH moiety could directly act to promote axon growth and its potency to promote axon growth could thus differ from that of the endogenous 15 kDa and 16 kDa isoforms previously detected in the E7 neural retina (24). Alternatively, this biological activity could reflect the action of 15 kDa GH, as exogenous recombinant 22 kDa GH can be endogenously cleaved into 15 kDa moiety.

A previous study has investigated the effects of various growth factors alone or in combination, in depolarizing conditions or not, on immunopanned RGC neurite growth in cells purified from E7 chick embryo (34). None of the growth factors studied (BDNF, CNTF, EGF, bFGF, GDNF, IGF-1, NGF, NT-3, NT-4, BBG) could induce a significant increase in process regrowth compared to control in cultures of "mature" RGCs selected by their increased adhesiveness to Thy-1 dishes. Preliminary studies from this group suggested, however, that BDNF could promote neurite outgrowth in depolarizing conditions (to elevate cAMP levels) in "immature" or loose bound cells compared to "mature" or tight bound cells. We thus confirm here that BDNF in association with forskolin promotes process (axon) outgrowth. In another study, Goldberg and colleagues (21) found that GH (10 ng/ml in the presence of 5 μM forskolin) slightly induced RGC axon elongation (by 17% +/- 7.3 in comparison with that induced by BDNF and forskolin (100%) and 4% +/- 2 in the controls) in postnatal day (P) 8 rats. We also detected a modest but significant increase in axon growth after GH treatment at the lowest dose examined (22 ng/ml, 10^{-9} M), although the different experimental conditions precludes any rigorous comparison with this earlier study. In contrast, a recent study demonstrated that GH does not affect neurite outgrowth of stem cell culture extracted from adult rat subventricular zone (23). This result is however not surprising, since GH negatively regulates the neuronal differentiation of these cells (7). The difference with the effect observed in the present study might reflect cell type (cortical vs RGC) and stage (adult vs embryonic) -related differences.

GH, as well as BDNF and forskolin, increased the length of small and long axons (> 200 μm) while RGC mostly produced small axons (< 40 μm) in absence of treatment. It is possible that GH stimulated different populations of RGCs, including one or more of the seven morphological types of RGC reported in the chick (35), or RGCs of different stages of maturation. GH might preferentially affect axonogenesis and / or axon growth in RGCs prior to their reaching their main target, the optic tectum, since GH is present as early as E4 in nascent RGC axons before the first RGC axon attain the tectum (at E6)

(35). It is thus of interest that "immature" E7 immunopanned RGCs are more responsive to growth factor treatment in chicks than "mature" RGCs (34) and that P8 rat RGCs are less responsive to trophic factors than E20 RGCs (20).

The mechanism through which GH could promote axon elongation may include microtubule polymerization, which is crucial for axon growth (36), as GH has been reported to stimulate tubulin polymerization in Chinese Hamster Ovary cells stably transfected with the rat GHR (37).

Does GH promote axon growth of other developing neurons?

GH treatment has been previously reported to induce an increase in neurite length and number in rat E17 cortical cells in culture upon GH treatment, although no quantification was performed (14). A reduction in fiber number is also observed in the corpus callosum of Snell dwarf mice, characterized by a deficit in GH, TSH and prolactin due to pit-1 mutation (38, 39).

GH immunoreactivity is distributed in other projecting axons of the developing nervous system. GH is found in one of the optic tectum projecting tract, the tectobulbar tract in early chick embryos (40). GH has also been found in the trigeminal, vagal, and spinal nerves in E7 chick embryos (3). GHR in turn has been detected in spinal motoneurons and splanchnic nerves in E40 bovine embryos (13). GH ability to promote axon growth in the long projecting axons of the retina might thus be extended to other projecting neurons of the developing CNS and peripheral nervous system (PNS).

Does GH promote axon growth in an autocrine manner?

Like GH (24), GHR mRNA is detected in cells of the GCL. In addition, GH and GHR colocalize in the OFL of E7 embryonic chicks. GH and GHR immunoreactivity observed in the OFL is thus likely to result from intra-RGC translation. GH induction of axon growth is thus likely to reflect local autocrine action of GH, especially as GH is not present in the pituitary or systemic circulation at these embryonic stages (25, 26). RGCs could thus use GH to autoregulate its own axon elongation.

It is also apparent that some of the GH and GHR immunoreactivity in the RGCs was present within nuclear compartments and their colocalization suggests GH may bind to GHR within RGC nuclei. Nuclear GH has been previously reported in many tissues (Rezvani et al., 1973; Harvey et al., 2000, 2001). Nuclear GHRs are well described for

GH (Lobie et al., 1994; Lincoln et al., 1998) and are more abundant than those in the plasma membrane or in cytosolic compartments (Fraser and Harvey, 1992). The presence of GHRs in the nucleus may reflect their role in the translocation of GH from the cell surface to the nucleus, as observed for many other growth factors (Smalheiser, 1996). The roles of GH and other growth factors within the nuclear compartment are, however, unknown.

Does GH participate in the ontogenic loss of axon growth?

RGCs irreversibly lose their ability to grow their axons during development (20). This intrinsic loss is thought to be triggered by a contact-mediated or membrane-associated signal emanating from adjacent amacrine cells, at least in neonate rats (20). In chick embryos, an ontogenic decrease in axon growth has been observed *in vitro* on laminin substrate, as E6 RGC axons can grow on laminin, while E11 RGCs can no longer do so (22). As GH detection within the OFL decreases after E12, GH might be associated with RGC loss of responsiveness to laminin substratum and to its loss of axon growth ability in general. This is further supported by the fact that exogenous GH has been suggested to induce nerve regrowth in adult PNS nerves (41-43) which do not lose their ability to elongate if severed. GH treatment induces regeneration of a previously lesioned sciatic nerve in healthy and hypophysectomized rats, as measured by "the pinch-test" (41). GH treatment also promotes an increase in the number of nitric oxide synthase (NOS)-containing penile nerve fibers and improves electrostimulated erectile function following lateral cavernous nerve neurotomy or bilateral cavernous nerve grafting (42, 43). In addition, axon regeneration in mouse dorsal root ganglion neurons depends on JAK / STAT, the main molecules involved in GH signaling (44). The ontogenic downregulation of GH in RGCs might thus be associated with RGC loss of axon growth ability while its upregulation might lead to axon regeneration in severed nerves.

The irreversible loss of RGC axon growth is also associated with a concomitant increase in dendrite growth (20). This switch is similarly thought to be induced by a retinal signal (20). The fact that GH is present in the inner plexiform layer (IPL) at E12-E18, where RGC dendrites make connections with amacrine and bipolar cells, suggests GH might also promote dendrite growth and thus participate in this switch. It is thus of interest that neurons of the motor cortex of Snell dwarf mice and Little mice [characterized by absent circulating GH due to a mutation in the GHRH receptor gene (45, 46)] display an impaired dendritic growth (39) and that growth of dendrites is restored to normal upon administration of exogenous bovine GH (bGH) in association with thyroxine within 20 days after birth in Snell mice (47, 48). GH might thus undergo a differential trafficking (from RGC axons at E4-E12, to RGC dendrites at E12-E18) to locally promote axon then dendrite outgrowth according to the cell needs.

In conclusion, GH is present in RGC axons during their elongation to their target and promotes RGC axon growth.

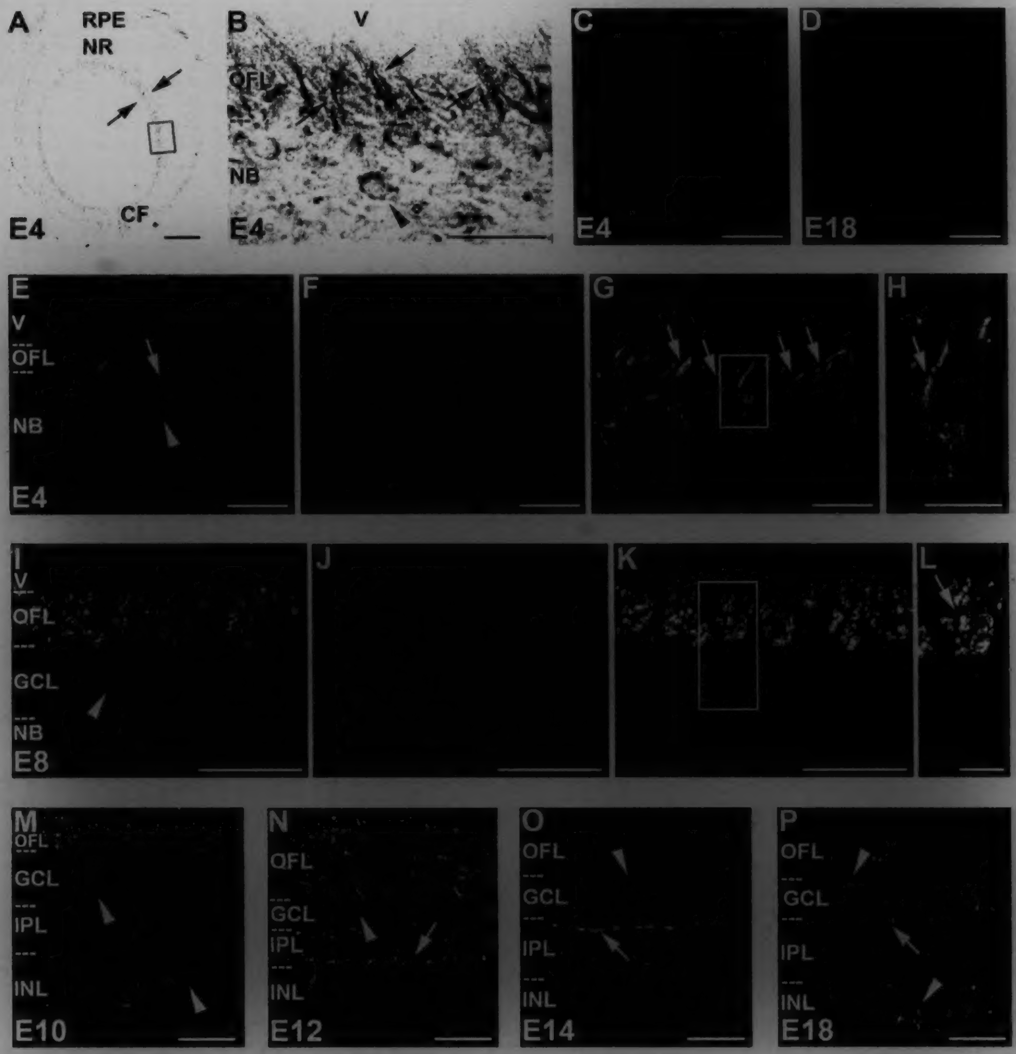
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Fig. 4.1. GH immunoreactivity in the neural retina throughout embryogenesis. **(A)** Brightfield microscopy showing GH-immunoreactivity (brown) in the E4 eye in a parasagittal plane (NR, neural retina; RPE, retinal pigmented epithelium; CF, choroid fissure). The nascent optic fibre layer is located between the arrows. **(B)** Higher magnification of the boxed area in (A). Note the GH-immunoreactivity in fascicles of the optic fibre layer (OFL) (arrows) and in the cytoplasm of the subjacent large, rounded cells (arrowhead) (NB, neuroblastic layer). **(C)** Negative (normal rabbit and mouse sera) control for confocal GH and neurofilament associated protein staining in the E4 chick embryo. **(D)** Negative control (normal rabbit serum) for fluorescent GH staining in the E18 embryo. **(E)** Fluorescent GH staining (green) in the cytoplasm and nuclei (arrowhead) of cells adjacent to the OFL and in neurites emanating from these cells (arrow) in the E4 chick embryo (v, vitreous). **(F)** Neurofilament-associated protein immunoreactivity (red) in the OFL of the E4 chick retina. **(G)** Image overlay of (E) and (F) showing colocalization of GH and neurofilament-associated protein (arrows). **(H)** Higher magnification of the boxed area in (G). **(I)** GH immunoreactivity in the OFL and ganglion cell layer (GCL) of the E8 neural retina. Note the presence of GH in cytoplasm and nucleus of cells in the GCL (arrowhead). **(J)** Neurofilament-associated protein immunoreactivity in the OFL of the E8 chick embryo. **(K)** Image overlay of (I) and (J), showing colocalization (yellow-orange coloration) in the OFL. **(L)** Higher magnification of the boxed area shown in (L). Note GH and neurofilament-associated protein colocalization in a fascicle of the OFL (arrow). **(M)** GH immunoreactivity in the OFL and cells of the GCL and inner nuclear layer (INL) (arrows) at E10. Note the absence of staining in the inner plexiform layer (IPL). **(N)** GH immunoreactivity in the OFL, in cells of the GCL (arrowhead) and in the IPL (arrowhead) at E12. **(O)** GH immunoreactivity in the OFL, in cells of the GCL (arrow) and in the IPL (arrowhead) at E14. **(P)** GH-immunoreactivity in cells of the GCL and INL (arrowhead) and in the IPL. Note the absence of signal in the OFL. Scale bars = 20 μm except for A = 50 μm, H = 10 μm and L = 5 μm

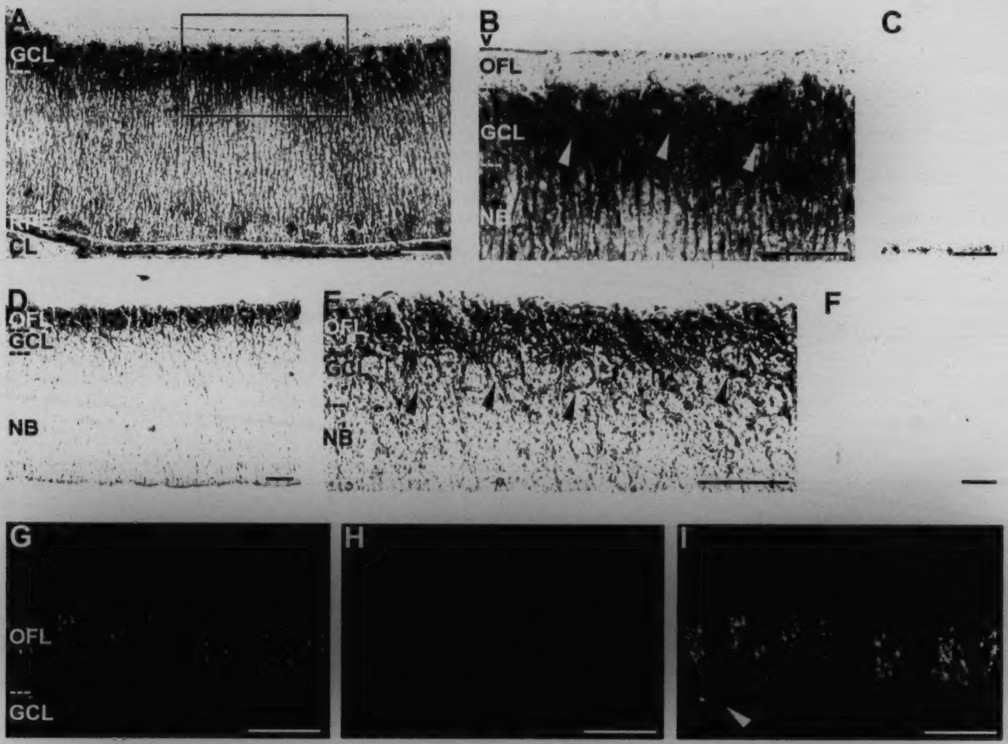

Fig. 4.2. GH receptor (GHR) immunoreactivity and mRNA in the neural retina of E7 chick embryos. (**A**) *In situ* hybridization using a Dig-labeled antisense riboprobe complementary to the GHR mRNA (GCL, ganglion cell layer; RPE, retinal pigmented epithelium; NB, neuroblastic layer; CL, choroid layer). (**B**) Higher magnification of the boxed area in (A). Note the strong staining in cells of the GCL (arrowheads) (v, vitreous). (**C**) Absence of labeling when the antisense probe is replaced by the sense probe. (**D**) Brightfield microscopy showing GHR immunoreactivity (brown) in the neural retina of E7 embryonic chicks. (**E**) Neural retina at higher magnification showing GHR immunoreactivity in the innermost part of neural retina in fascicles of the OFL (arrows) and cytoplasm of cell in the GCL (arrowheads). (**F**) Negative (normal mouse serum) control. Confocal microscopy showing GH (green) (**G**) and GHR (red) (**H**) immunoreactivities in the OFL of an E7 chick embryo retina. (**I**) Image overlay of (H) and (I) showing colocalization (yellow-orange coloration) in the OFL and in the cytoplasm and nucleus of a representative cell in the GCL (arrowhead). Scale bars A-F = 20 μm and G-I = 10 μm

Fig. 4.3. Immunopanned retinal ganglion cells (RGCs). (**A**) RGC purified by immunopanning and immunoreactive for GH. (**B, C**) High magnification of these cells showing immunoreactivity in the nucleus (arrowhead) and in the neurites (arrow) of theses cells. (**D**) Absence of signal when the primary antibody is replaced by NRS. Scale bar = 20 μm

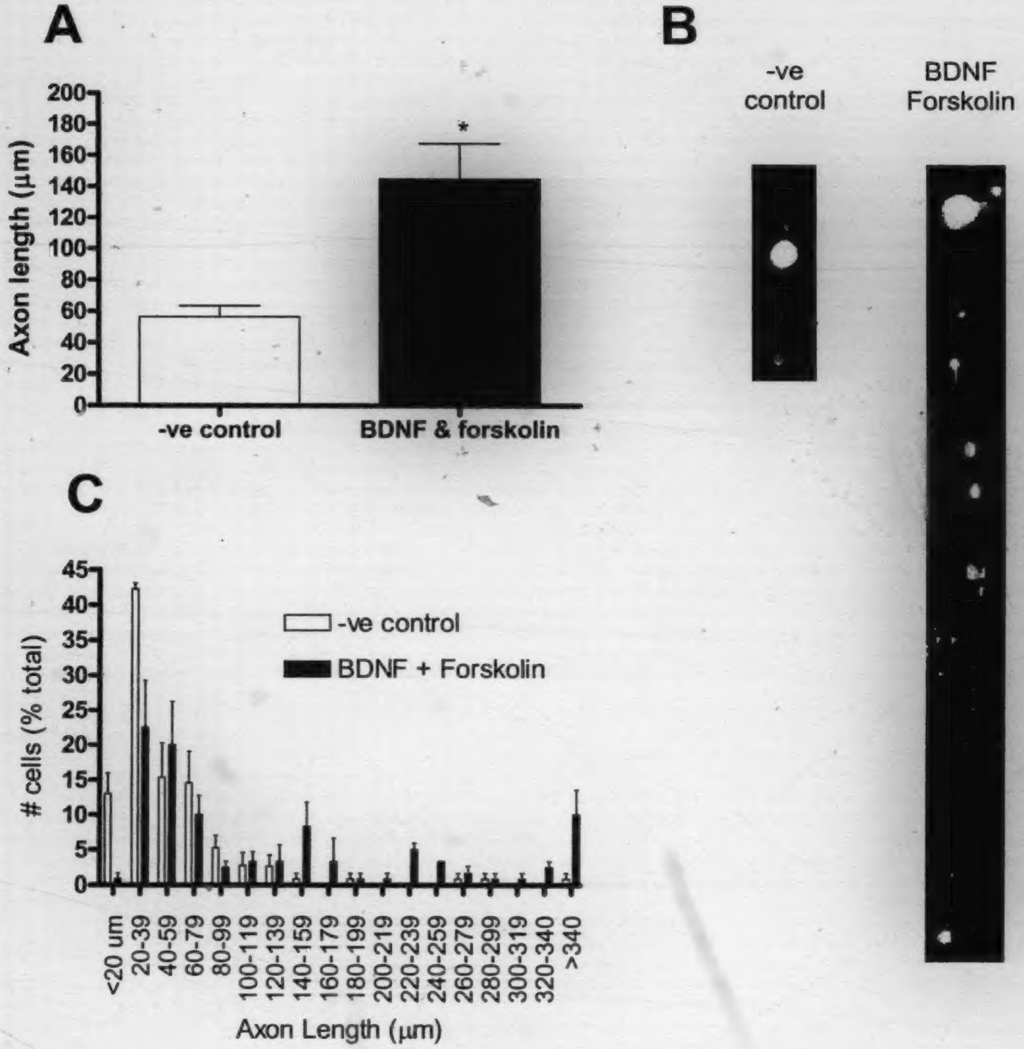

Fig. 4.4. Retinal ganglion cell (RGC) responsiveness to treatment. (**A**) Average axon length of immunopanned RGCs in absence or presence of 50 ng/ml BDNF and 10 µM forskolin after three days in culture. Values are means + S.E.M. (*n*=4 dishes). * = $P < 0.05$ in comparison to the PBS control (unpaired *t*-test). (**B**) Two illustrative RGC neurons stained with DiI. Note the increased axon length upon treatment. (**C**) Profile of axon length. Note the increase in proportion of longer axon upon treatment (Mean + SEM).

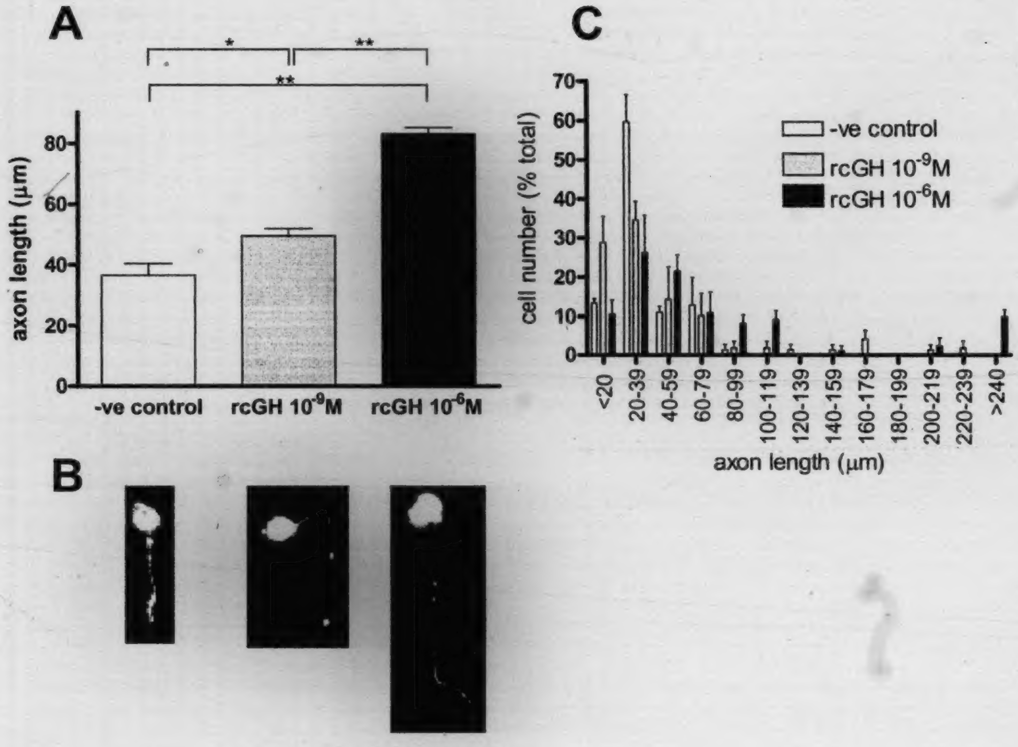

Fig. 4.5. Retinal ganglion cell (RGC) axon elongation upon treatment with exogenous rcGH. (**A**) Effect of 10^{-9}M and 10^{-6}M rcGH treatment upon axon elongation in RGC immunopanned in 3 day cultures. Values are means + S.E.M. (*n*=3 dishes). * = $P < 0.05$ and ** = $P < 0.001$ (One-way analysis of variance followed by Tukey's Multiple Comparison Test). (**B**) Illustrative RGCs in absence or presence of rcGH treatment. (**C**) Proportion of cells elongating axons of specific length in absence or presence of treatment (mean + SEM).

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Chapter 5

Expression, Translation and Localization of a Novel, Small Growth Hormone Variant

Chapter 5: Expression, Translation and Localization of a Novel, Small Growth Hormone Variant[1]

5.1 Introduction

In higher vertebrates, the growth hormone (GH) gene is comprised of five exons (1 to 5) and four introns (A to D). Full-length GH mRNA is formed from the 5 spliced exons and is translated into a 22 kDa protein (1). Alternative exon splicing does, however, result in a heterogeneity of GH transcripts (2). A 45 bp deletion at the 5' end of exon 3 is, for instance, present in the GH mRNA from human (3,4) and rat (5) pituitary glands and the resulting transcript codes for a 20 kDa protein. Alternative splicing within exon 3, at a different site, similarly results in a transcript with a 73 bp deletion (6). Similar variants are also present in GH mRNA (hGH-V) in the human placenta and testis (7-9). Alternative splicing of GH mRNA in the placenta results in a transcript with a 4 bp deletion at the end of exon 4 and codes for a different protein (hGH-V3) (7-11). Human GH (hGH) mRNA may also have deletion of exon 3, deletions of exons 3 and 4 or deletions of exons 2, 3 and 4 (12,13). An exon 3-deleted GH variant has also been shown to be translated *in vitro* (13).

Other GH variants have also been described that result from intron retentions. For instance, an intron D retaining variant is present in human (13) and bovine (14) pituitary glands. The retention of this intron results in a shift in the reading frame and is likely to produce an isohormone with a C-terminus that differs from that in full-length GH (14). As this isoform exists in polysomes (14), it is likely to be translated. Similarly hGH-V mRNA retains intron D and likely encodes a 26 kDa protein with a different C-terminus to 22 kDa hGH-V (4,10).

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[1] A version of this chapter has been published: Baudet ML, Martin BT, Hassanali Z, Parker E, Sanders EJ and Harvey S Expression, translation and localization of a novel, small growth hormone variant *Endocrinology.* 2007 **148**: 103-15 . BT Martin (Fig. 5.10, 5.12-14) and Z Hassanali (Fig. 5.8E-I) assisted with the immunohistochemistry and E Parker (Dr. EJ Sanders's laboratory) performed the western blotting (except for Fig. 5.5A, 5.6A,B and scGH expression plasmid synthesis that I carried out), immunocytochemistry and immunoneutralization experiments.

Another GH transcript has also been identified in mRNA from the eye and heart of embryonic chicks (15). This transcript is not, however, the product of alternative splicing *per se*, but the product of an alternate promoter. This novel transcript, small chicken GH (scGH, 15) is severely truncated in that it lacks exons 1-3 of the full-length chicken GH gene and retains part of intron C. If translated, it is thought to code for a 16.5 kDa protein.

Besides pituitary 20 kDa GH, no GH variants have, however, been clearly shown to be translated and/or to have biological activities. As Takeuchi *et al* (15) provided no evidence for the presence of scGH in chick embryos, the possible translation of this novel transcript was investigated in the present study.

5.2 Materials and Methods

Tissues

Since Takeuchi *et al* (15) had identified scGH mRNA in the eye and heart of early embryos by 5'rapid amplification of cDNA ends, whole eyes, the dissected neural retina (NR), the heart, and the heartless bodies of chick embryos at embryonic day (E) 7 of the 21-d incubation period were rapidly fixed for immunohistochemistry or placed into RNA later for subsequent RNA extraction (Ambion, Austin, Texas). For comparison, whole pituitary glands from slaughterhouse fowl were similarly collected.

RT-PCR.

Total RNA was extracted from tissues using RNeasy Mini or Midikits (Qiagen, Burlington, Ontario,Canada) and mRNA obtained using Oligotex mRNA Minikits (Qiagen). Total RNA (0.1-5μg) or mRNA (0.165μg) were reverse transcribed (for 50 min at 50°C) into cDNA using 10 U/μl Superscript III RNAse H^-, 0.025 μg/μl oligo $(dT)_{12-18}$ or 0.1μg/μl anchored oligo $(dT)_{20}$, 5mM dithiothreitol (DTT), 1x First Strand Buffer and 1mM each deoxynucleotide triphosphates (dNTPs) (all from Invitrogen, Burlington, Ontario,Canada). RNA templates were digested (20 min at 37 °C) using RNAse H, and cDNA was amplified with 0.1 U/μl Platinum *Taq* DNA Polymerase High Fidelity, 2 mM $MgSO_4$, 1x High Fidelity PCR buffer, 0.2 mM each dNTPs (all from Invitrogen) in the presence of 0.2 μM each forward and reverse oligonucleotide primers designed to amplify a 426 bp fragment covering the entire coding sequence of scGH mRNA (forward primer, scGH-F3: 5'-GGCATGCAGCAGCACTGCAG-3'; reverse primer, scGH-R2: 5'-AGAGAGCAACTGCACCATCTGA-3') (Fig. 5.1A). The primers were designed using OligoPerfect software (Invitrogen) and synthesized by Sigma Genosys (Oakville, Ontario, Canada). The initial denaturation was at 94 °C for 2 min and was followed by 38 cycles of PCR (94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 68 °C for 30 s) and a final extension, for 10 min. PCR products were visualized on 0.4 μg/ml ethidium bromide gels.

Cloning

Each PCR fragment was gel-extracted using QiaQuick Gel extraction kits (Qiagen). Extracted PCR products (4 μl) were then ligated with 10 ng pCR-II TOPO vector (Invitrogen) in 1.2 μmol NaCl and 0.06 μmol $MgCl_2$ (5 min at room temperature). Then, 2 μl of this solution were mixed with One Shot TOP10 (Invitrogen) chemically competent cells and incubated (30 min, on ice). The transformed cells were then heat-shocked (42 °C for 30 sec) and incubated (37 °C for 1h) with SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). The transformed cells were then streaked on Luria Bertani (LB) (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) agar (15 g/liter) plates that had been coated with 1.6 mg X-gal (Rose Scientific, Edmonton, Alberta, Canada) containing ampicillin (100 μg/ml) or kanamycin (50 μg/ml). The streaked plates were then incubated (37 °C, overnight) to obtain individual colonies. Six colonies were selected and grown (37 °C, overnight) in 20 ml LB medium containing ampicillin (100 μg/ml) or kanamycin (50 μg/ml). Plasmids were then extracted using QiaPrep Spin Miniprep kits (Qiagen). The ligation of the PCR products was checked by gel electrophoresis of 1μg plasmid after digestion with 20 U EcoR1 restriction endonuclease, in 1x EcoR1 buffer (New England Biolabs, Pickering, Ontario, Canada) and its orientation verified after amplification of 0.1 μg plasmid in a PCR reaction [0.02 U/μl Platinium Taq DNA polymerase, 2 mM $MgCl_2$, 1x PCR buffer, 0.2 mM each dNTPs, in the presence of 0.2 μM each forward scGH-F3 and reverse (M13F (-20), 5' GTAAAACGACGGCCAGT 3'] primers. The PCR consisted of 2 min of denaturation at 94 °C, and then 38 cycles of denaturation (94 °C for 30 sec), annealing (60 °C for 30 sec) and extension (72 °C for 30 sec), followed by a final period (10 min) of extension (at 72 °C).

Sequencing

Gel extracted plasmids were sequenced commercially (Macrogen Inc., Seoul, Korea).

scGH Expression

Plasmid synthesis. scGH cDNA was obtained from whole eyes E7 embryos, as described above. The PCR fragment was gel extracted and 4 μl of the pure product was ligated (5 min at room temperature) to 10 ng pcDNA 3.1 / V5-His TOPO TA Vector (Invitrogen) in 1.2 μmol NaCl and 0.06 μmol $MgCl_2$. As the last three bases of the reverse primer correspond to a stop codon, the V5 epitope and the polyhistidine tag are not expressed. The plasmid was then transfected and subcloned, as described above. Ampicillin LB plates and medium was used to grow colonies. The ligation and orientation was validated by PCR analysis, using forward T7 (5' TAATACGACTCACTATAGGG 3') and reverse scGH-R2 primers. One hundred percent homology with the published sequence of scGH (15) was found.

Expression. The scGH expression plasmid (Fig. 5.1B) was transfected into human embryonic kidney (HEK) 293 cells (kindly donated by Dr. J.R. Casey, Department of Physiology, University of Alberta,Edmonton, Canada), using lipofectamine 2000 (Invitrogen). In brief, HEK 293 cells were cultured onto 10 μg/ml poly-D-lysine (Sigma) coated 24 well. After a 24 hour incubation period (37 °C, 5% CO_2), transfection was performed; 1.6 μg of expression plasmid and 4 μl of Lipofectamine 2000 were mixed with Opti-MEM I, according to manufacturer's instructions, then the complex was added to each well containing cells at 85% confluence in 500 μl DMEM, 5% fetal bovine serum, 5% fetal calf serum, 2 mM L-glutamine (all Invitrogen), without antibiotics. After a 48h culture, the cells and medium were collected separately. Cells were scraped from the wells, centrifuged (200 x g, 5 min) to remove any residual medium then lysed by sonication in 100 μl of a protease inhibitor cocktail (Roche-Applied-Science, Laval, Québec, Canada). The medium was similarly centrifuged twice (200 x g , 5 min) to remove any contaminating cells and the cell-free medium was concentrated 25 fold using a centrifugal filter unit (Amicon ultra-15, Millipore, Cambridge, Ontario, Canada) . Protein content in the media and lysates were determined by the Bio-Rad method (Bio-

Rad Laboratories, Mississauga, Ontario,Canada) and aliquots of the samples were analysed by Western blotting.

scGH antisera

The unique N-terminus of scGH, encoded by the nucleotides derived from intron C of the full-length GH gene (Fig. 5.1C), was synthesized commercially (Washington Biotechnology Inc. Columbia, Maryland, USA) (to >96% purity). This 20 amino acid peptide (MQQHCRTPHLHSSEIPLSFQ) was conjugated with keyhole limpet hemocyanin and the conjugate was then used to raise antisera in New Zealand White Rabbits (Washington Biotechnology). Three months after initial immunization and 10 days after the last of 3 booster injections of the conjugate, terminal blood samples were collected from the rabbits and serum was stored at -80 °C. The titre of the antiserum (SH-1) was determined by ELISA (Washington Biotechnology) using the unconjugated peptide as the ligand. At 50% inhibition of binding, unpurified serum had a titre of approximately 1:8000 (Fig. 5.2).

Western Blotting

Tissues were collected into a protease inhibitor solution [HEPES, $MgCl_2$, EDTA, EGTA, aprotinin, leupeptin and pepstatin in 1% (wt/vol) phenylmethylsulfonylfluoride] and homogenized. After centrifugation and protein determination by the Bradford dye-binding procedure (Bio-Rad Laboratories, Mississauga, Ontario, Canada), the supernatants were stored at -20 °C before Western analysis. Samples (containing 20-100 µg protein) were analyzed by one-dimensional SDS-PAGE (16). The samples were added to loading buffer 10% (wt/vol) glycerol, 5% (wt/vol) β-mercaptoethanol, 2% sodium dodecyl sulfate, 0.001% (wt/vol) bromphenolblue (pH 6.8) and denatured at 100 °C for 4 min and the proteins separated by electrophoresis in 12% gels. After electrophoresis, the proteins were equilibrated in transfer buffer [25 mM Tris, 192 mM glycine, 20% (wt/vol) methanol] and transferred electrophoretically (100V for 1h at 4 °C) to nitrocellulose paper (Bio-Rad Laboratories). After transfer, nonspecific binding sites were blocked with 5% (wt/vol) nonfat dry milk in Tris-buffered saline (20 mM Tris HCl, 0.5 M NaCl, pH 7.6), containing Tween 20 for 1h at room temperature. scGH immunoreactivity was detected using SH-1, diluted 1:1000 in Tris-buffered saline (TBS) /5% (wt/vol) non-fat dry milk. After an overnight incubation at room temperature, the membranes were washed with TBS containing Tween 20 (3 x 10 min) and then incubated for 1h at room temperature with a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, California, USA; 1:5000). Antibody binding was then visualized using Avidin biotin Complex (ABC) reagents (Vector Laboratories) 1h at room temperature, and after washing, the blots were developed with an enhanced chemiluminescence detection system (Enhanced Chemiluminescence Kit, Amersham , Piscataway,NJ) and exposed to X-AR film (Kodak, Rochester, New York, USA). The specificity of labeling was determined by pre-incubating (3h at room temperature) the primary antibody with excess (100 μg/ml) N-terminal scGH (Washington Biotechnology) or purified chicken pituitary GH [National Hormone and Peptide Program, (NHPP) Torrance, California, USA; 100 μg/ml]. Pre-immune rabbit serum (normal rabbit serum, NRS) was also used as a negative control.

Immunohistochemistry

Fixation. Tissues were fixed in freshly prepared Carnoy's Fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid), overnight at 4°C. They were then washed in PBS (2x15min) and dehydrated in a graded series of ethanol (50%, 2x15min; 70%, 30 min; 95%, 30min; 100%, 2x30min) and cleared with Hemo-De (Fisher Scientific, Edmonton, Alberta, Canada) for 30 min. Tissues were then infiltrated with paraffin wax overnight at 60°C under normal atmospheric pressure. Serial transverse (6μm) sections were taken using a microtome and mounted onto charged slides (Fisher Scientific).

Brightfield microscopy. Immunohistochemical staining was performed with commercial reagents (Vector Laboratories) using the ABC method (17). Sections were cleared in Hemo-De (2x5 min), rehydrated in a graded series of ethanol (100%, 2x5 min; 95%, 2 min; 70%, 2 min; 50%, 2 min; distilled water, 2 min), equilibrated in PBS (2 min), and then incubated in H_2O_2 (1% in 50% methanol) to block endogenous peroxidase. The sections were washed in PBS (3x5 min) then incubated in PBS containing 10% normal goat serum (NGS) (Sigma, Oakville, Ontario, Canada) and 0.4% Tween 20 for 1 hour. The slides were subsequently incubated overnight at 4°C with the scGH antibody (SH-1), diluted 1:4000 in 1% goat serum. After incubation, the slides were washed (3 x 5 min) in PBS and incubated for 1h at room temperature in biotinylated goat anti-rabbit IgG (Sigma, 1:500). The slides were then washed in PBS (3 x5 min) and incubated in ABC reagent for 1h at room temperature and washed again. Staining was visualized using the chromogenic substrate diaminobenzidine tetrahydrochloride (DAB; Sigma), which resulted in a brown coloration. For comparison adjacent sections were also stained using a specific polyclonal antibody raised in rabbits against native chicken (c) GH (αcGH-1); (18), diluted 1:000 in PBS. Preabsorption of the primary antibodies, using excess (100 μg/ml) N-terminal scGH (Washington Biotechnology) or purified cGH (NHPP) provided negative controls. Other controls included the omission of the secondary antibody and the replacement of the primary antibodies with PBS.

Confocal microscopy. The sections were similarly dehydrated, blocked with 10% Normal Goat Serum (NGS) (in PBS containing 0.4% Tween 20) for 1 hour and incubated overnight at 4°C with the primary antibodies (SH-1 at 1:4000; αcGH1 at 1:1000) for the colocalization of scGH and cGH, respectively. An anti-islet 1 mouse monoclonal antibody (39,4 D5, undiluted ; Developmental Studies Hybridoma Bank at the University of Iowa, Ames, IA) was also used for the localization of retinal ganglion cells (RGCs) (16). Glial progenitor cells were labeled using a monoclonal antibody against vimentin (H5, Developmental Studies Hybridoma Bank at the University of Iowa, at 1:20 dilution) (19,20). A mouse monoclonal antibody against chick neurofilament-associated antigen (3A10, at1:500 dilution; Developmental Studies Hybridoma Bank at the University of Iowa,) was also used to detect neural fibres (21,22). The possibility that scGH might be localized within the nucleolus of RGCs was also examined using a specific marker (SYTO RNA Select, Invitrogen, at 500 mM in PBS dilution) (23). After incubation with the rabbit antibodies, the sections were incubated for 1h at room temperature in goat anti-rabbit IgG conjugated to Alexa Fluor 488 ($F(ab')_2$ fragment, Invitrogen), at a dilution of 1:500. The sections were then washed (3 x 10 min) and mounted with Gel mount (Biomeda, Foster City, CA). For colocalization studies with monoclonal antibodies, an additional one hour incubation was performed before mounting with goat anti-mouse IgG conjugated to Alexa Fluor 555 ($F(ab')_2$ fragment, Invitrogen) at a dilution of 1:500, followed by PBS washes (3 x 5 min). The labeled sections were examined using a LSM 510 confocal microscope (Carl Zeiss, Göttingen, Germany), equipped with appropriate lasers.

Retinal ganglion cell immunopanning

RGC immunopanning. To confirm the presence of scGH in RGCs, these were isolated by an immunopanning technique, using a monoclonal antibody to the chick Thy-1 antigen (24). Immunopanning substrata were prepared by incubating coverslips with 50 μg/ml goat anti-mouse IgG in sterile Tris buffer (pH 9.5), for 12 h at 4 °C. Coverslips were washed three times with 0.25% BSA in PBS, and incubated with 55 μg/ml anti-chick Thy-1 antibody (gift of Dr. P.L. Jeffrey, University of Sydney, Australia) for 2 h at room temperature. Coverslips were then washed three times with PBS, blocked with 0.25% BSA in PBS for 15 min at room temperature, and then washed again three times with PBS.

Retinas from four embryos at E8 were dissected and placed in an Eppendorf tube in calcium- and magnesium-free Tyrode's solution. The tissue was centrifuged and 600 μl of 0.1% trypsin (Sigma) were added to the tube at 37 °C for 10 min. Inactivation of the trypsin was accomplished by adding fetal bovine serum (FBS; Invitrogen) to a final concentration of 10%, together with 1% BSA, and the mixture was gently pipetted to dissociate the cells. Ten units of DNase I (Sigma) were added, and the cells were centrifuged at 15,000 rpm for 2 min. The pellet was washed with 10% FBS in Tyrode's solution and the cells were centrifuged again. The supernatant was then removed and the cells were re-suspended in Medium 199 (Invitrogen) with 10% FBS and gentamycin. The cell suspension was incubated on anti-Thy-1 coated coverslips for 1.5 h at room temperature. The coverslips were washed five times with Tyrode's solution to remove non-adherent cells. We have previously shown that more than 90% of the adherent cells resulting from this procedure are RGCs (24,25).

For immunocytochemistry, the adherent cells were first dislodged from the anti-Thy-1-coated coverslips and re-plated on coverslips coated with poly-L-lysine (Sigma) in Petri dishes (26). Cells were released from the anti-Thy-1 coating by incubating them with 2.5% trypsin (Sigma) in Earle's balanced salt solution, containing bicarbonate and phenol red, but without calcium and magnesium (Sigma), for 10 min at 37 °C. The trypsin was then inactivated by adding 1ml Medium 199 containing 30% FBS and the cells were removed from the coverslip using fine jets of medium from a pulled pipette.

When all the cells had been removed, the suspension was transferred to an Eppendorf tube containing 100µl of 100% FBS. The cell suspension was then centrifuged at 15,000 rpm for 2 min, the supernatant was discarded, and the resulting pellet was re-suspended in Medium 199 containing 30% FBS. This suspension was placed in drops onto coverslips coated with poly-lysine. Coverslips bearing the cultures were washed with warm Medium 199 and fixed with 4% phosphate buffered paraformaldehyde. Cultures were then permeabilized with acetone at -20 °C and non-specific immunoreactivity was blocked with 4% BSA in PBS. The cells were then labeled with the scGH antiserum and mounted in Vectashield (Vector Laboratories). Negative controls were carried out by replacing the scGH antibody with NRS. The labeled cells were examined using a Zeiss LSM 510 confocal microscope equipped with appropriate lasers.

scGH Immunoneutralization

The possibility that scGH in immunopanned RGCs might have local actions in cell survival, similar to the antiapoptotic actions of full-length cGH (24,25), was investigated by its immunoneutralization. Immunopanned RGCs were resuspended in 150µl Medium 199 containing 30% FBS. This suspension was placed in drops onto coverslips coated with poly-lysine in Petri dishes and incubated in a humid chamber, overnight at 37 °C in the presence or absence of the scGH antiserum, at a final dilution of 1:100.

Apoptosis in the RGC-enriched cultures was assessed, as before (24,25) by labeling cells with 4', 6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, Oregon, USA) to show shrunken and fragmented apoptotic nuclei and counting the number of apoptotic nuclei in random microscope fields of 9 control and 9 experimental cultures. Statistical differences in the data were determined by Student's *t* test.

5.3 Results

RT-PCR

In the presence of oligonucleotide primers scGH-F3 and scGH-R2, a 426 bp cDNA of expected length was amplified from reverse-transcribed mRNA extracted from the whole eye and heart of E7 embryos (Fig. 5.3A). This fragment was also amplified from reverse-transcribed mRNA from the heartless bodies of these embryos and from the dissected neural retina of E7 eyes (Fig. 5.3A). When amplified with scGH-F3 and scGH-R2, this 426 bp fragment was also abundantly generated from reverse-transcribed mRNA from the pituitaries of slaughterhouse fowl (Fig. 5.3A). In contrast, this fragment was not amplified from any tissue when mRNA was used as the template, nor in non-template controls (NTC), in which DNA was replaced by RNAse / DNAse- free water (Fig. 5.3A). When sequenced (Fig. 5.3B), each amplicon had 100% homology to the published coding sequence for scGH (15).

scGH Western Blotting

Western blotting of proteins extracted from the whole retina (neural retina and the retinal pigmented epithelium, RPE) using the scGH antibody (SH-1) detected immunoreactivity associated with a protein of 31 kDa and, to a lesser extent, a protein of 16 kDa (Fig. 5.4). Another protein of approximately 29 kDa was also detected with weaker immunoreactivity (Fig. 5.4). Similar immunoreactive bands of 31 kDa were detected (Fig. 5.5A) in proteins from the whole eye (lane 1), the neural retina (lane 2), the RPE (lane 3), cornea (lane 5) and lens (lane 6), although not in some samples of vitreous humor (Fig. 5.5A, lane 4). When the blots were overexposed (Fig. 5.5B), immunoreactivity for a 16 kDa protein was also present in all ocular tissues. The scGH-immunoreactivity in the whole eye and in the neural retina was completely lost after the preabsorption of the primary antibody with excess N-terminal scGH (Fig. 5.5A, lane 7 and Fig. 5.5B, lane 6). In addition to ocular tissues, scGH immunoreactivity was also detected in the pituitary glands of slaughterhouse chickens, in which proteins of 31 kDa (Fig. 5.5B, lane 5; Fig 5.6A, lane l), and 16 kDa (Fig. 5.5B, lane 5) were readily detected.

This 31 kDa protein was similarly detected in different samples of the pituitary, RPE, NR and lens (Fig. 5.6A, lanes 1, 3, 4 and 6) and was additionally present in the choroid (Fig. 5.6A, lane 2) and in a different sample of the vitreous humor (Fig. 5.6A, lane 5). This protein was not, however, detected when the same ocular tissue extracts were probed with NRS (Fig. 5.6A, lane 7) or the antibody raised against pituitary GH (αcGH-1), which detected a moiety of approximately 25 kDa in the RPE, vitreous and lens (Fig. 5.6B, lanes 3, 5 and 6). This protein is the most abundant GH moiety in the pituitary gland (Fig. 5.6B, lane1), although smaller proteins, including 15 kDa and 16 kDa moieties, were additionally detected in pituitary extracts (Fig. 5.6B, lane 1) and the choroid, RPE, NR and lens (Fig. 5.6B, lanes 2, 3, 4 and 6). Interestingly, whereas a 15 kDa moiety in the vitreous was immunoreactive with αcGH-1 (Fig. 5.6B, lane 5), the 16 kDa moiety was not detected in vitreous humor (Fig. 5.6B, lane 5). Proteins of 15 and 25 kDa are therefore specifically labeled by αcGH-1, whereas SH-1 specifically detects a 31 kDa moiety and, when the blots are overexposed, a 16 kDa protein. The differential specificity of these antisera is also shown in Fig. 5.6C, in which the scGH antibody does not detect the full length (25 kDa) GH protein. Moreover, preincubation of the scGH antibody with excess cGH (100 μg/ml) does not impair its labeling of the 31 kDa protein in the neural retina of E7 chick embryo eyes, which is completely blocked when the antibody is preabsorbed with excess (100 μg/ml) N-terminal scGH.

The specificity of the scGH antibody is also demonstrated by its ability to label N-terminal scGH applied to 8% acrylamide gels (Fig. 5.7A, lanes 1 and 2), although under the conditions used, the apparent molecular size of this peptide (<5 kDa) could not be determined accurately. This immunoreactivity was completely lost after the preabsorption of the scGH antibody with excess (100μg/ml) N-terminal scGH (Fig. 5.7A, lane 5). Figure 5.7 also shows translation of the scGH transcript in HEK cells transfected with the scGH expression plasmid since 31 kDa and 16 kDa scGH immunoreactivity is present in lysates of these cells (Fig. 5.7A, lane 3). The scGH immunoreactivity of both of these proteins was completely lost when the scGH antibody was preabsorbed with excess N-terminal scGH (Fig. 5.7A, lane 6). In contrast with the cell lysates, scGH immunoreactivity was not seen in the medium in which the transfected HEK cells were cultured (Fig. 5.7A, lane 4). Figure 5.7B also shows that the scGH immunoreactivity in lysates of the transfected HEK cells (lane2) was identical in size to that in the neural retina of E7 embryos (lane2).

Immunohistochemistry

scGH immunoreactivity was readily detectable in the anterior eye (Fig. 5.8A), notably in the cytoplasm of cells in the corneal epithelium (Fig. 5.8C, E, F), the developing ciliary body (Fig. 5.8A) and the lens epithelium (Fig. 5.8D, G, H). This immunoreactivity was lost when the primary antibody was preabsorbed with excess N-terminal scGH (Fig. 5.8B) or replaced with NRS (Fig. 5.8I).

scGH immunoreactivity was also present at the back of the eye (Fig. 5.9A), particularly in the neural retina in which it was widespread, but also in the endothelium of blood vessels in the choroid (Fig. 5.9B). It was not, however, present in the optic fibre layer (OFL) nor in the optic nerve head ONH (Fig. 5.9A-C). The specificity of the staining in the neural retina is indicated by its absence when the primary antibody was replaced with NRS (Fig. 5.9D). As expected (16), immunoreactivity for full-length GH was present in the neural retina, but primarily in the OFL (Fig. 5.9E, F), comprised of axons from underlying retinal ganglion cells (RGCs). This immunoreactivity was also intense in the ONH (Fig. 5.9F, G), in which axons from the RGC fasciculate to form the optic nerve. The absence of scGH immunoreactivity in the OFL was clearly shown by confocal microscopy with a neurofilament antibody (Fig. 5.10). Neurofilaments were abundantly present in the fibers comprising the OFL (Fig. 5.10B), and confocal imaging demonstrated that scGH (Fig. 5.10A) and neurofilaments were not colocalized in this structure (Fig. 5.10C). Confocal microscopy localized scGH immunoreactivity within the nucleus and cytoplasm of cells in the neural retina (Fig. 5.10A and 5.10E). Some of these cells were identified as RGCs, using a RGC nuclear marker (39.4D5) (Fig. 5.10F). The localization of scGH in the nucleus of RGCs was indicated by the resulting yellow-orange coloration (Fig. 5.10G). Staining was detected within the nuclei of many RGCs indicating the presence of scGH immunoreactivity in a discrete nuclear subcompartment. This subcompartment was not the nucleolus, because scGH immunoreactivity was not colocalized with a nucleolus-specific marker (data not shown). The specificity of staining for scGH and for the RGC marker was shown by the absence of fluorescence in NRS-treated control sections (Fig. 5.10D, H). The presence of scGH immunoreactivity in the cytoplasm and nucleus of RGCs was also demonstrated by the labeling of Thy-1 immunopanned E8 RGCs (Fig. 5.11A), whereas NRS controls had no specific fluorescence (Fig. 5.11B).

Fluorescence microscopy of the ONH clearly showed the presence of full-length GH immunoreactivity in discrete fibre tracts (Fig. 5.12B), which lacked scGH immunoreactivity (Fig. 5.12A). This finding was confirmed by the observation that scGH immunoreactivity in the ONH (Fig. 5.12C) was not colocalized in the fibre tracts (Fig. 5.12E) that were strongly immunoreactive for the neurofilament antibody (Fig.

5.12D). Control sections treated with NRS had no fluorescence (Fig. 5.12F). Transverse sections through the optic nerve (Fig. 5.13) demonstrated scGH fluorescence in concentric tissue layers of the sheath surrounding the optic nerve (Fig. 5.13A), which was immunoreactive for neurofilament (Fig. 5.13B). Confocal imaging clearly showed that the immunoreactivity for scGH was largely confined to this sheath and not present in the ensheathed nerve (Fig. 5.13C). In similar sections, the scGH immunoreactivity detected (Fig. 5.13E) was in cells that were also stained with the vimentin antibody (Fig 5.13F), as revealed by the yellow-orange coloration in these presumptive progenitor glial cells when the images were overlaid (Fig. 5.13G). Control sections (Fig. 5.13D, H) treated with NRS showed no fluorescence.

In extra-ocular tissues, scGH immunoreactivity was also abundant in the anterior pituitary gland of slaughterhouse chickens, in which it was present in both the caudal and cephalic lobes (Fig. 5.14A, B). In marked contrast, immunoreactivity for full-length GH was, as expected, predominantly located in the caudal lobe (Fig. 5.14B, E). For comparison, no signal was in the caudal and cephalic lobes ydetected with the primary antibodies were replaced by normal serum (Fig. 5.14C, F).

Immunoneutralization

In preliminary studies, the immunoneutralization of endogenous scGH in immunopanned RGCs increased ($P<0.05$) the number of DAPI-labeled apoptotic nuclei in comparison with untreated controls (Fig. 5.15).

5.4 Discussion

These results confirm the presence of novel scGH transcript in the heart and eyes of embryonic chicks, in agreement with Takeuchi *et al* (15), and show that it is particularly abundant in the neural retina. We also show that it is expressed in the pituitary glands of 6-7 week old (slaughterhouse) birds. This transcript is therefore expressed in tissues that also express the full-length GH gene (16,27,28).

The cGH gene has only one TATA Box, in the 5' untranslated region, and scGH transcription starts downstream of this, within intron C of the full-length cGH gene. It is therefore likely that scGH transcription is initiated by a different promoter to that regulating the transcription of the full-length gene. This possibility is supported by the presence of 4 different transcription initiator elements (TIEs) (tcagtccc, tactttt, tcacaccc, tcatattc) in intron C, upstream of the scGH transcription start site (15). This possibility has yet to be assessed, but it would be a novel discovery, because only one promoter has previously been associated with GH gene transcription (29). An alternative TATA-less promoter located within a downstream intron that induces the transcription of 5' truncated (hCATL B) variant has, however, been discovered for the human cathepsin L gene (30). Similarly, ptd-FGFR4 is a truncated isoform of the fibroblast growth factor receptor (FGFR) 4 gene, which is transcribed by an alternate TATA Box containing promoter located within its intron 4 (31). Alternate promoters within intron sequences have also been described for the human sodium bicarbonate cotransporter (NBC)-1 gene (32) and for the human α-fetoprotein gene (33).

Our finding of scGH mRNA in the neural retina suggests that it is a source of scGH in ocular tissues. In contrast, Takeuchi *et al* (15) could not detect this transcript in dissected ocular tissues (choroid , neural retina, retinal pigmented epithelium, sclera, recten, lens, vitreous body, iris, ciliary body and cornea) and therefore hypothesized that the scGH mRNA they detected in the whole eye was derived from nonocular tissues, such as peripheral blood cells or extraocular muscle. This difference may, however, reflect our use of a different oligonucleotide primer set for PCR.

Expression of the scGH transcript was achieved in HEK transfected cells, as demonstrated by the presence of scGH immunoreactivity in cell lysates. Most of the scGH immunoreactivity detected was associated with a protein of approximately 31 kDa, although a protein of approximately 16 kDa was also present. The predicted size of scGH is 16.5 kDa (15), this strongly suggests that monomer scGH is predominantly dimerized by bonds that are resistant to Sodium Dodecyl Sulfate (SDS) and β-mercaptoethanol disruption. It is therefore of interest that a 45 kDa dimer of 22 kDa GH has been described that is held together by interchain disulfide bonds that are exceptionally resistant to reducing agents and thus confers extreme stability to the homodimer (34). Similar bonds may hold the scGH homodimer together, because scGH retains the four cysteine residues of monomeric (25 kDa) cGH (at positions 5, 113, 130 and 138 of scGH), which also exists in SDS-and β-mercaptoethanol resistant dimerized isoforms of pituitary GHs (35).

Dimerization through the linkage of intermolecular cysteine residues by disulfide bonds is thought to protect GH from proteolytic degradation, without impairing its ability to bind membrane GH receptors (GHRs) (34). Although scGH lacks many of the residues thought to be required for binding to membrane GHRs, it is predicted to have the same C-terminal sequence and secondary structure as full-length GH (15) and may bind to the chicken GHR. This possibility is supported by the presence of GH immunoreactivity in the chicken eye with the molecular size of a putative scGH-GH binding protein (GHBP) complex (15). The possibility that scGH has biological activity is also supported by our preliminary studies that show that endogenous scGH immunoneutralization in cultured RGCs promotes cell death, similar to that induced by the immunoneutralization of the full-length molecule (24,25). Functional studies on the roles of scGH are, however, precluded by the current unavailability of the purified protein.

Although scGH immunoreactivity was present in the lysates of scGH-transfected HEK cells, it was not present in the incubation medium. This is consistent with the lack of a signal peptide in the predicted scGH sequence (15), indicating that it is not a secreted protein. Furthermore, the hydropathy profile of scGH indicates it has a hydrophilic N-terminus that is also unlikely to serve as a signal peptide (15). If it has biological activity, it must therefore act as an intracrine within the cytosol or within nuclear compartments. It is therefore relevant that in the third helical domain of scGH, leucine residues are arranged every 7 amino acids, which is a characteristic of leucine zipper transcription factors with nuclear action (15).

In contrast to ocular tissues, 31 kDa scGH immunoreactivity was mostly absent from vitreous humor. This further indicates that it is not a secretory protein. Its presence in some samples of vitreous likely reflects its cellular compartment and contamination with cells from the retina, lens, cornea or ciliary body. The absence of scGH in the vitreous humor contrasts with the abundance of immunoreactivity detected by the antibody raised against native pituitary GH (16,18). This immunoreactivity detected by αcGH-1 is associated with monomer (25 kDa) GH and with a 15 kDa moiety, which is likely to be a proteolytic fragment (36). It is also of interest that this antibody did not detect the 16 kDa moiety in vitreous humor that was detected in other ocular tissues and the pituitary gland. This finding is consistent with previous observations (16,18), which led to speculation that this 16 kDa moiety might be scGH. However, as αcGH-1 does not detect a 31 kDa protein, which is likely to be dimerized scGH and more abundant than monomer scGH, the 16 kDa protein detected by this antibody is unlikely to be scGH.

The presence of scGH immunoreactivity in the transfected HEK cells demonstrates the translation of this novel GH transcript. The translation of this variant likely occurs in frame with the full-length monomer. The truncated GH variant has five atg (start) codons, of which four are in frame with full-length cGH. The other atg codon in the scGH sequence would only encode a peptide of 19 amino acids, much smaller in size than scGH and the proteins detected by Western blotting. Taken together, this suggests that scGH and cGH are translated in frame.

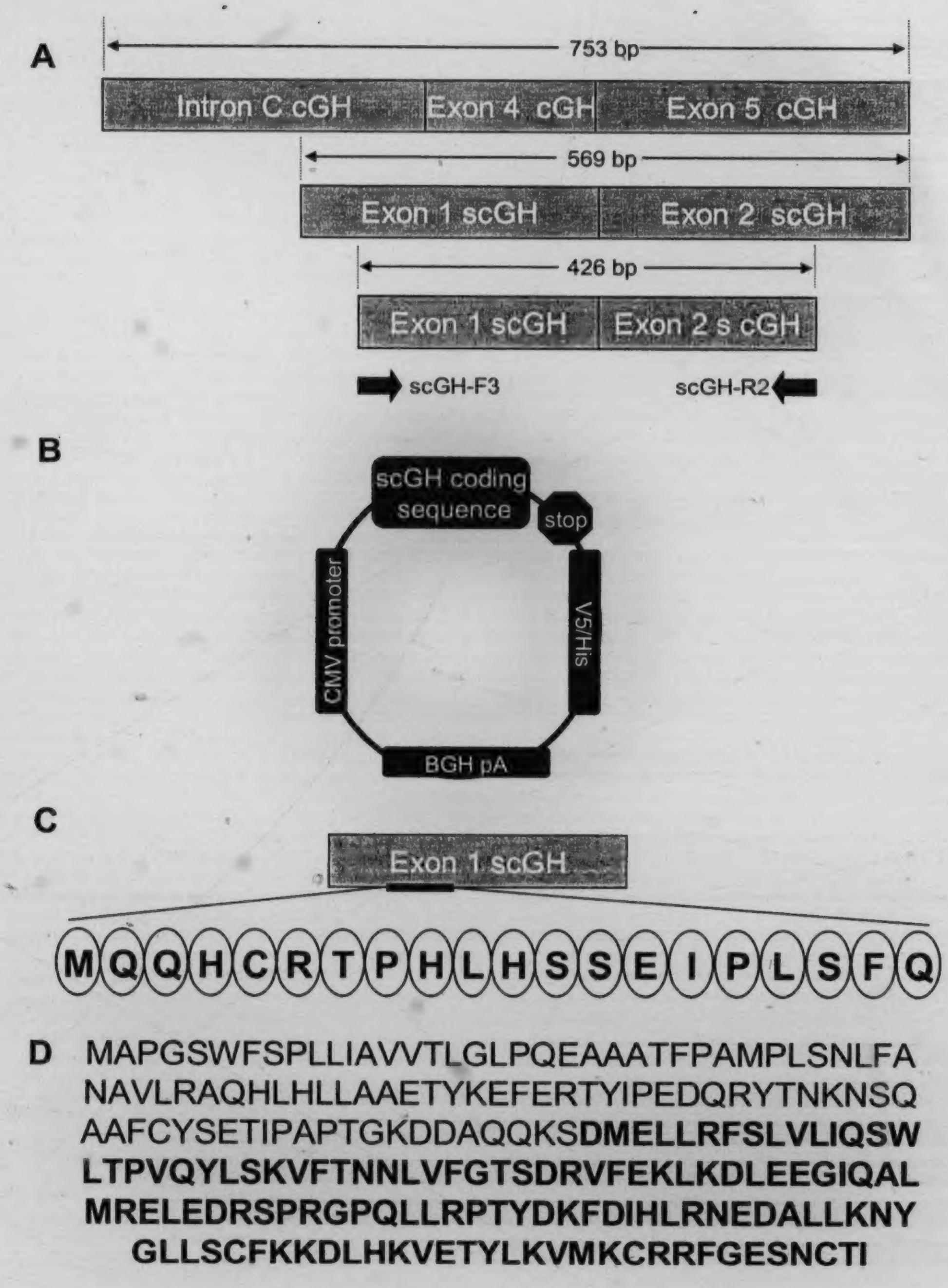
The translation of the variant was similarly demonstrated by the widespread presence of scGH immunoreactivity in ocular tissues of chick embryos. This is the first time the scGH protein has been localized, because previous immunocytochemical studies have used specific antibodies against full-length chicken GH (16,18) or rat GH (15). The antibody that we generated (SH-1) was instead directed against the unique N-terminus of scGH, which is not present in any other protein in the National Center for Biotechnology Information (NCBI) protein database. The specificity of the antibody is also demonstrated by its inability to detect full-length chicken GH and by the complete loss of immunological staining after the preincubation of the primary antibody with N-terminal scGH and by its ability to detect the predicted 16.5 kDa protein.

Within the E7 eye, immunoreactivity for scGH was detected in the lens and corneal epithelium, the presumptive ciliary body, and the neural retina, particularly within RGCs. Immunoreactivity for full-length chicken GH, is also present in these tissues, as previously reported using αcGH-1 as the primary antibody (16,18). This suggests that both GH transcripts are expressed and translated in these occular tissues. However, while scGH immunoreactivity is clearly present in the RGCs of the neural retina, it was not present in RGC axons in the OFL nor in the fibre tracts in the ONH. This is in marked contrast to the distribution of GH immunoreactivity detected by αcGH-1. This finding suggests the different GH moieties may undergo differential intracellular trafficking. This is of interest, especially as the absence of scGH in neural fibres further suggests it does not enter into secretory pathways for release from nerve terminals. This finding is therefore similar to the differential intracellular trafficking, secretion and endosomal localization of two interleukin (IL)-15 isoforms (37), one of which (a truncated variant) remains in the cytoplasm and is not secreted. The intracellular location and organelle association of scGH should therefore be further determined.

Within the retina, immunoreactivity for scGH was present in both the cytoplasmic and nuclear compartments of the RGCs. Indeed, it was particularly prominent within a nuclear sub-compartment that was not labeled by the nuclear RGC marker, nor by a marker of the nucleolus. The identity of this compartment and the role of scGH in the nucleus is, however, unknown. GH immunoreactivity has previously been localized in the nucleus of chick (16) and rat (38) RGCs, although full-length GH immunoreactivity in the embryonic chick RPE was thought to be primarily cytoplasmic (39). In other tissues, GH immunoreactivity has also been found in nuclear compartments, specifically in the nucleoplasm (in the eu-heterochromatin junction) as well as in the inner and outer nuclear membranes (40-43). The presence of GH and scGH in the nucleus is consistent with their putative roles as intracrines and may reflect the nuclear localization of GHRs (40,41,43,44) and nuclear GH-binding sites (45,46). Although the sequence of scGH does not have a classical nuclear localization signal (NLS, as determined by PSORTII and Predict NLS software) (47,48), it could enter the nucleus through passive diffusion, because it is smaller than 50 kDa or bind to a chaperone protein that has a classical nuclear localization signal (49,50).

In addition to the RGCs of the neural retina, scGH immunoreactivity was found in vimentin-labeled progenitor glial cells ensheathing the optic nerve. Immunoreactivity for full-length GH has not been detected in this tissue, which suggests GH gene transcription may undergo tissue-specific regulation within the embryonic chick eye. Similarly, in extraocular tissues, scGH mRNA and protein were detected in the adenohypophysis of slaughterhouse chickens. However, whereas full-length GH is confined to somatotrophs in the caudal lobe (16,51,53), scGH immunoreactivity was abundantly present in both the caudal and cephalic lobes. scGH is unlikely to be secreted and therefore sequestered by cephalic lobe cells, which suggests that cell-specific regulation of GH gene transcription also occurs in the adenohypophysis. Cell-specific regulation of GH-transcripts has similarly been seen for members of the human GH gene family in the pituitary gland, placenta, testis and blood mononuclear cells (7, 10, 54), although it has yet to be described for any nonprimate species. This cell specificity may reflect cell-specific transcription factors like *Pit-1* that regulate full-length GH gene expression (55), although specific factors that regulate scGH expression have yet to be determined. The demonstration of two, translated, GH transcripts in the avian hypophysis is therefore a highly novel and exciting finding.

In summary, these results demonstrate, for the first time, that the transcript for the small GH variant is translated and document its distribution within ocular and extraocular tissues of chick embryos.


A
753 bp
Intron C cGH
Exon 4 cGH
Exon 5 cGH
569 bp
Exon 1 scGH
Exon 2 scGH
426 bp
Exon 1 scGH
Exon 2 s cGH
scGH-F3
scGH-R2
B
scGH coding sequence
stop
V5/His
BGH pA
CMV promoter
C
Exon 1 scGH
M Q Q H C R T P H L H S S E I P L S F Q

D MAPGSWFSPLLIAVVTLGLPQEAAATFPAMPLSNLFA
NAVLRAQHLHLLAAETYKEFERTYIPEDQRYTNKNSQ
AAFCYSETIPAPTGKDDAQQKS**DMELLRFSLVLIQSW**
LTPVQYLSKVFTNNLVFGTSDRVFEKLKDLEEGIQAL
MRELEDRSPRGPQLLRPTYDKFDIHLRNEDALLKNY
GLLSCFKKDLHKVETYLKVMKCRRFGESNCTI

Fig. 5.1. (**A**) Schematic representation of scGH exons that transcribe 569 bp scGH mRNA, in comparison with intron C and exons 4 and 5 of the full-length chicken GH gene. The 426bp coding region of the transcript can be amplified by RT-PCR using the oligonucleotide primer set scGH-F3 and scGH-R2. (**B**) Schematic representation of the scGH-expressing plasmid. The PCR product of the scGH coding sequence is inserted into a pcDNA3.1 / V5-His-TOPO plasmid vector. The expression of scGH is driven by a cytomegalovirus (CMV) promoter and is terminated at the bovine GH polyadenylation sequence (BGH pA). Because scGH coding sequence encodes for a stop codon (stop), the V5 epitope and polyhistidine tag (V5/His) are not translated. (**C**) Schematic representation of the 20 N-terminal aminoacids coded by exon 1 of scGH. (**D**) The complete amino acid sequence of the full-length cGH protein (Gene Bank AAQ 81586.1) is shown. Amino acids that are homologous to those in acGH (Gene Bank BAB 62262.1) are shown in bold.

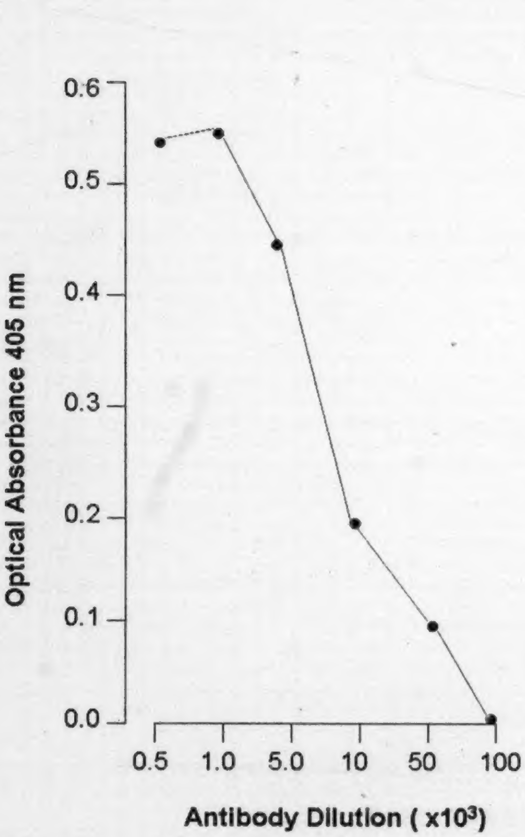
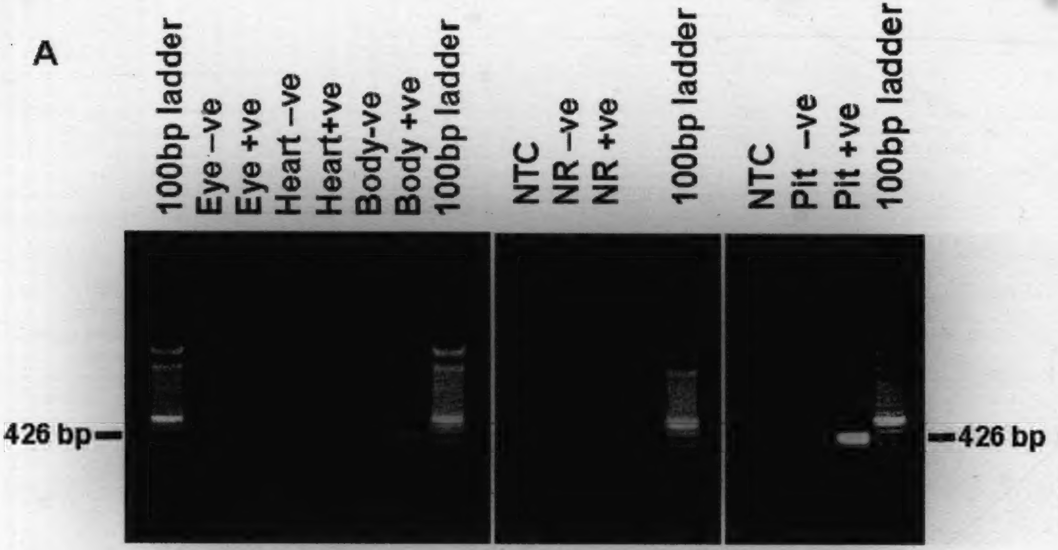


Fig. 5.2. Dilution curve for the scGH antibody (SH-1) determined by calorimetric ELISA (Washington Biochemicals). SH-1 (in a volume of 100µl) was incubated for 60 min at room temperature with unconjugated N-terminal scGH (at 2 µg/ml), which was adsorbed to the wells of a microtiter plate. After aspiration, a goat anti-rabbit antibody conjugated to horseradish peroxidase was added to the wells and incubated for 60 min. After aspiration and washing, an enzyme substrate 2,2-azino bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was added to each well and the color developed after 30 min was measured at 405 nm.



B

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scGH     1         GAGTGGTGGTGCTCAGCTTACAGAGCTGCCTCTGGGCTGCTTCAGGGAGAGCAG  54

Query    1  GGCATGCAGCAGCACTGCAGAACACCTCACCTGCACAGCTCTGAAATCCCTTTGTCATTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
scGH    55  GGCATGCAGCAGCACTGCAGAACACCTCACCTGCACAGCTCTGAAATCCCTTTGTCATTT  114

Query   61  CAGGACATGGAGCTGCTTCGGTTTTCACTGGTTCTCATCCAGTCCTGGCTCACCCCCGTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
scGH   115  CAGGACATGGAGCTGCTTCGGTTTTCACTGGTTCTCATCCAGTCCTGGCTCACCCCCGTG  174

Query  121  CAATACCTAAGCAAGGTGTTCACGAACAACTTGGTTTTTGGCACCTCAGACAGAGTGTTT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
scGH   175  CAATACCTAAGCAAGGTGTTCACGAACAACTTGGTTTTTGGCACCTCAGACAGAGTGTTT  234

Query  181  GAGAAACTAAAGGACCTGGAAGAAGGGATCCAAGCCCTGATGAGGGAGCTGGAGGACCGC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
scGH   235  GAGAAACTAAAGGACCTGGAAGAAGGGATCCAAGCCCTGATGAGGGAGCTGGAGGACCGC  294

Query  241  AGCCCGCGGGGCCCGCAGCTCCTCAGACCCACCTACGACAAGTTCGACATCCACCTGCGC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
scGH   295  AGCCCGCGGGGCCCGCAGCTCCTCAGACCCACCTACGACAAGTTCGACATCCACCTGCGC  354

Query  301  AACGAGGACGCCCTGCTGAAGAACTACGGCCTGCTGTCCTGCTTCAAGAAGGATCTGCAC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
scGH   355  AACGAGGACGCCCTGCTGAAGAACTACGGCCTGCTGTCCTGCTTCAAGAAGGATCTGCAC  414

Query  361  AAGGTGGAGACCTACCTGAAGGTGATGAAGTGCCGGCGCTTCGGAGAGAGCAACTGCACC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
scGH   415  AAGGTGGAGACCTACCTGAAGGTGATGAAGTGCCGGCGCTTCGGAGAGAGCAACTGCACC  474

Query  421  ATCTGA  426
            ||||||
scGH 475    ATCTGAGGCCCTGTGCCTGCGCCATGGCTGACGGCCCTGTCCCCCCCCCCCCCTTCCTC  534

SCGH 535    CCCGTCACCAAAAACACGAGGAATAAACCCCACAG 569
```

Fig. 5.3. (**A**) scGH mRNA detection by RT-PCR. Using the oligonucleotide primers scGH-F3 and scGH-R2, an ethidium bromide stained 426 bp fragment was amplified from reverse transcribed total mRNA extracted from the whole eye, heart, heartless body and neural retina (NR) of E7 chick embryos and the anterior pituitary glands (Pit) of slaughterhouse chickens. No signal was detected in negative controls lacking the reverse transcriptase (-), nor in nontemplate controls (NTC), in which cDNA was replaced with RNAse / DNAse- free water. (**B**) Each amplified cDNA was sequenced (Query) and had 100% homology with the published scGH sequence [Takeuchi *et al.* (15)].

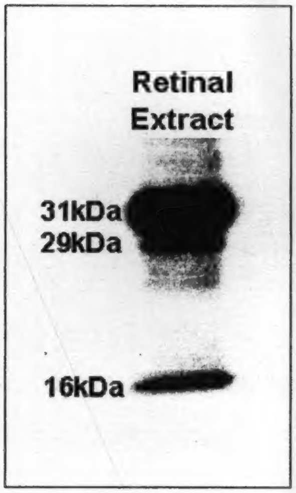
Retinal Extract
31kDa
29kDa
16kDa

Fig. 5.4. Western blotting of proteins extracted from whole retina of E7 chick embryos. Most of the immunoreactivity detected was associated with a 31 kDa protein, although proteins of 16 kDa and 29 kDa were also immunoreactive. Representative of at least six retinal extracts.

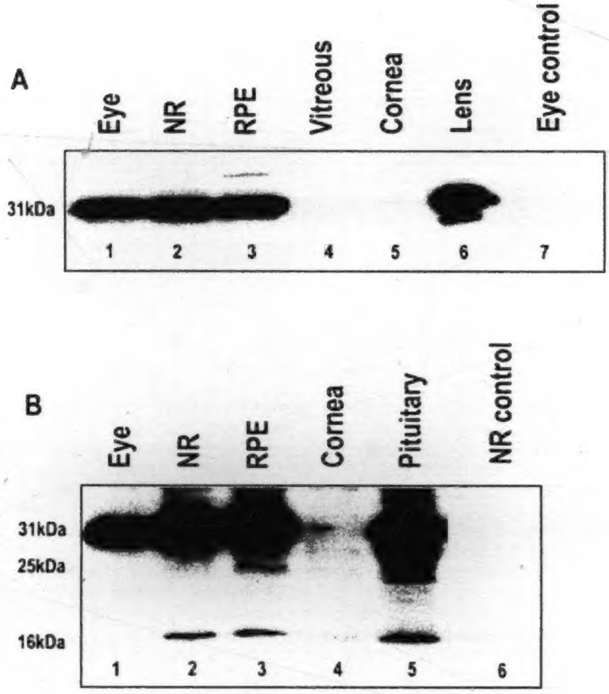
A
Eye
NR
RPE
Vitreous
Cornea
Lens
Eye control
31kDa
1
2
3
4
5
6
7
B
Eye
NR
RPE
Cornea
Pituitary
NR control
31kDa
25kDa
16kDa
1
2
3
4
5
6

Fig. 5.5. (**A, B**) Western blotting with the scGH antibody of proteins extracted from the whole eye, neural retina (NR), retinal pigmented epithelium (RPE), vitreous, cornea and lens of E7 chick embryos and from the anterior pituitary glands of slaughterhouse chickens. In (A) the blots were developed after an exposure period of 15 sec, whereas in (B) the exposure period was 2 min. The scGH immunoreactivity in the whole eye (A, lane 7) and in the neural retina (B, lane 6) was completely lost after the preabsorption of the primary antibody with excess N-terminal scGH. 20 μg protein was loaded in each lane. Representative of at least four similar blots.

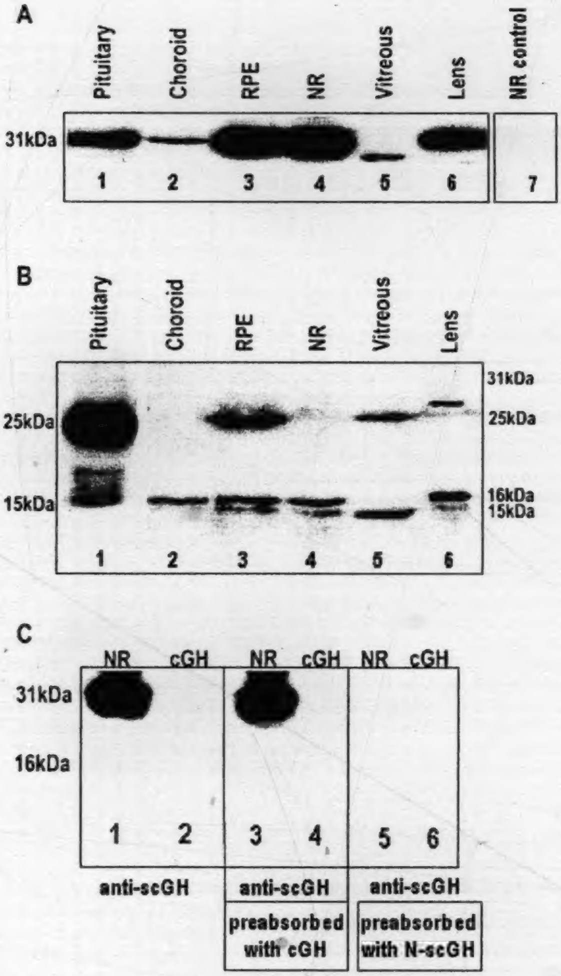
A
Pituitary
Choroid
RPE
NR
Vitreous
Lens
NR control
31kDa
1
2
3
4
5
6
7
B
Pituitary
Choroid
RPE
NR
Vitreous
Lens
25kDa
15kDa
31kDa
25kDa
16kDa
15kDa
1
2
3
4
5
6
C
NR
cGH
NR
cGH
NR
cGH
31kDa
16kDa
1
2
3
4
5
6
anti-scGH
anti-scGH
preabsorbed with cGH
anti-scGH
preabsorbed with N-scGH

Fig. 5.6. (**A**) Western blotting with the scGH antibody of proteins extracted from the choroid (lane 2), retinal pigmented epithelium (RPE) (lane 3), neural retina (NR) (lane 4), vitreous (lane 5) and lens (lane 6) of E7 chick embryos, in comparison with proteins extracted from the anterior pituitary glands of slaughterhouse chickens (lane 1) (20 µg protein loaded in each lane). A negative control, using normal rabbit serum (NRS) rather than the primary antibody is shown in lane 7. (**B**) Western blotting of the same tissue extracts with an antibody raised against native chicken pituitary GH, which primarily detects a 25 kDa moiety in the pituitary gland (lane 1, 1 µg of protein). (**C**) Western blotting with the scGH antibody of proteins extracted from the NR of E7 chick embryos (lanes 1, 3 and 5, 20 µg/lane), in comparison with purified chicken pituitary GH (lanes 2, 4 and 6, 10 µg/lane). In lanes 3 and 4, the primary antibody was preabsorbed with an excess of native pituitary GH (100 µg/ml, for 2h at room temperature) before use, whereas in lanes 5 and 6 the primary antibody was preabsorbed with an excess of N-terminal scGH (100 µg/ml, for 2h at room temperature) before use. This figure is representative of at least 3 similar blots.

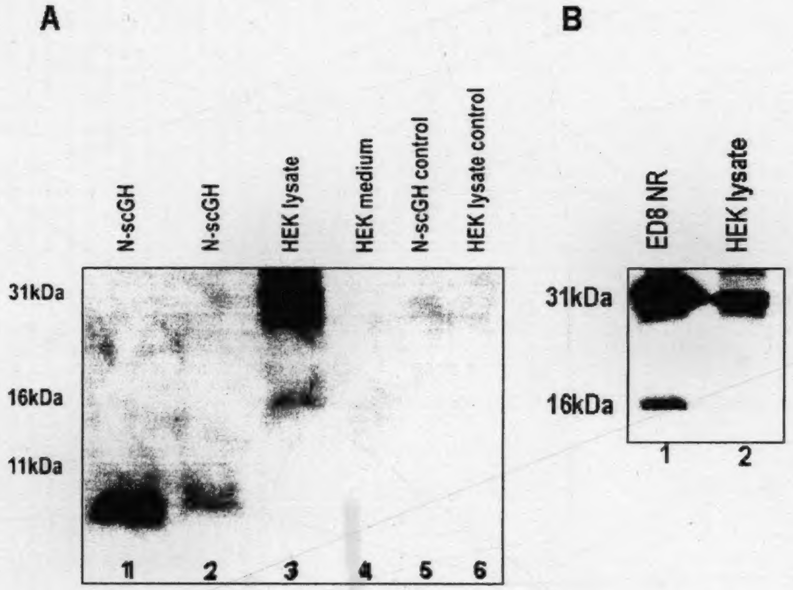
A
B
N-scGH
N-scGH
HEK lysate
HEK medium
N-scGH control
HEK lysate control
31kDa
16kDa
11kDa
1
2
3
4
5
6
ED8 NR
HEK lysate
31kDa
16kDa
1
2

Fig. 5.7. (**A**) Western blotting with the scGH antibody of proteins expressed in HEK 293 cells transfected with the scGH expressing plasmid (lane 3), in comparison with proteins present in the incubation medium (lane 4). The specificity of the antiserum is shown by its ability to detect N-terminal scGH (10 µg/ml, lane 1, and 5 µg/ml, lane 2) and by the complete loss of immunoreactivity when the antiserum was preabsorbed with N-terminal scGH (100µg/ml for 2h at room temperature) before use (lanes 5 and 6). (**B**) Western blotting with the scGH antibody of proteins expressed in HEK 293 cells transfected with the scGH expressing plasmid (lane 2) in comparison with proteins present in an extract of the neural retina of E8 chick embryos (lane 1).

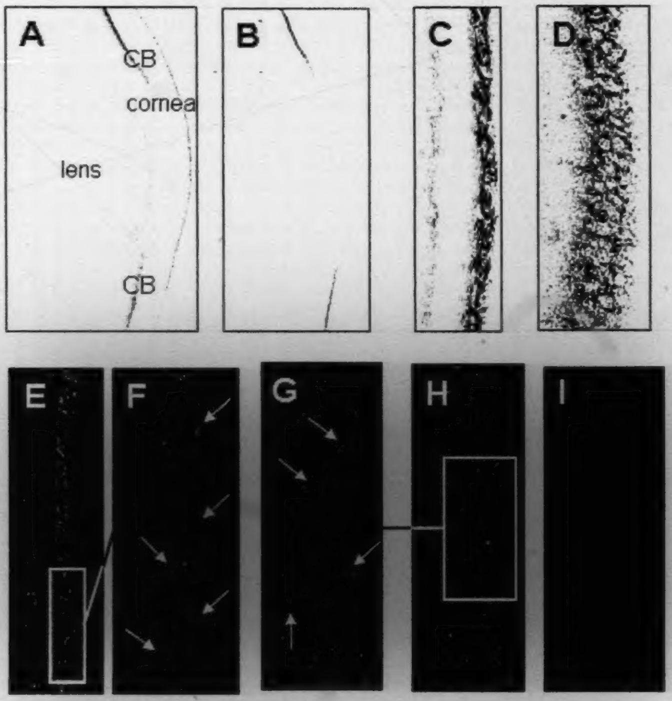

Fig. 5.8. scGH immunohistochemistry of the anterior eye of E7 chick embryos. **(A)** Brightfield microscopy of scGH immunoreactivity (brown staining) in the cornea (front of the lens), lens epithelium and ciliary body (CB) (Magnification x 400). **(B)** The specificity of this staining is shown using the same antibody after it had been preabsorbed with excess N-terminal scGH (100µg/ml for 2h at room temperature). Only the pigmented neural retina is visible after preabsorption (Magnification x 400). **(C)** scGH immunoreactivity in the inner and outer epithelial layers of the cornea (Magnification x 1000). **(D)** scGH immunoreactivity in the lens epithelium (Magnification x 1000). **(E-H)** Fluorescence microscopy of scGH immunoreactivity in the cytoplasm of cells (*arrows*) in the corneal epithelium (E-F) [Magnification x 400 (E), x 1800 (F)] and in the lens epithelium (G-H) [magnification x 400 (H), x 1800 (G)]. **(I)** No signal is present when the primary antibody was replaced with normal rabbit serum (Magnification x 400). Representative of similar sections from at least five embryos.

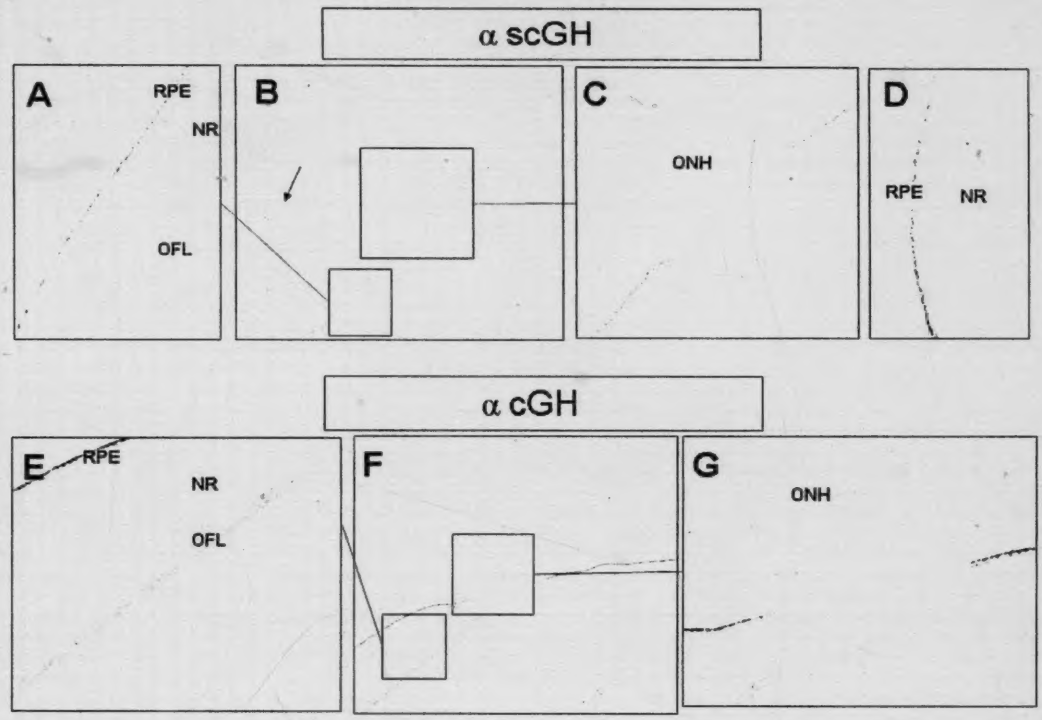

Fig. 5.9. scGH immunocytochemistry of the posterior eye of E7 chick embryos. (**A-C**) Brightfield microscopy of diffuse scGH immunoreactivity (brown) in the neural retina (NR) (RPE, retinal pigmented epithelium). Note the absence of staining in the optic fibre layer (OFL) (A-C) and the optic nerve head (ONH) (C). scGH immunoreactivity is also present in endothelial cells of blood vessels in the choroid (*arrows*) (B). (**D**) A control section treated with normal rabbit serum (NRS) to show the specificity of staining. Immunoreactivity for native (full-length) pituitary GH is also shown for comparison (**E-G**). Note the intense labeling of the OFL (E and F) and ONH (F and G) (Magnification x 100 in each case).

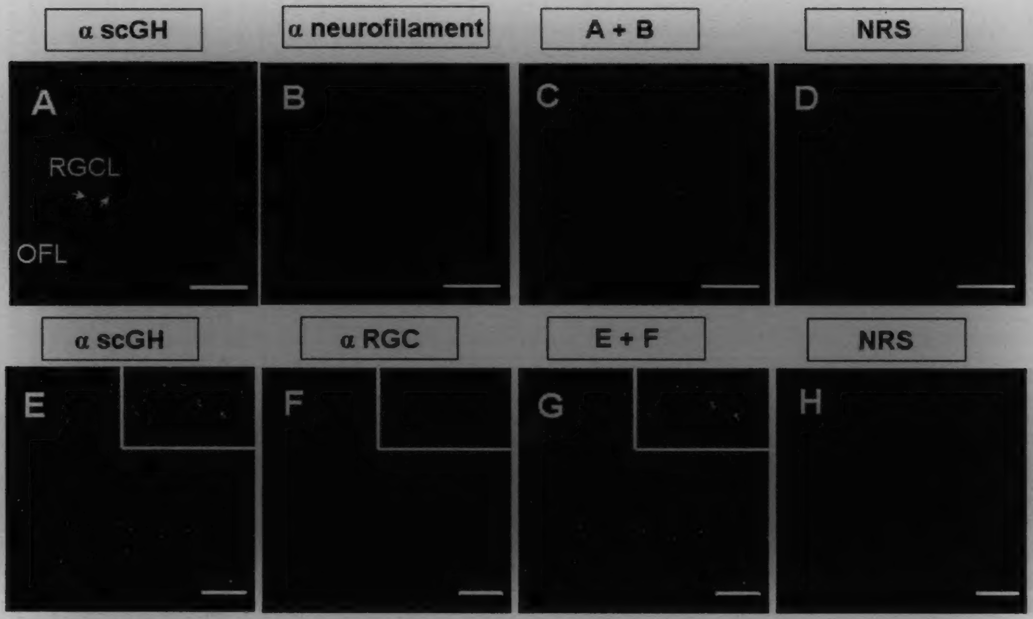

Fig. 5.10. Confocal microscopy of scGH immunoreactivity in the neural retina of E7 chick embryos. (**A**) scGH immunoreactivity *(arrows)* is seen in the cytoplasm of cells in the retinal ganglion cells layer (RGCL). No scGH immunoreactivity is present in the optic fibre layer (OFL). Staining for neurofilament immunoreactivity is shown in (**B**), and is confined to the OFL. (**C**) The image overlay clearly shows that scGH immunoreactivity and neurofilament immunoreactivity are discrete. (**D**) The specificity of detection is shown in a control, normal rabbit serum (NRS) section. The immunoreactivity for scGH in the neural retina (**E**) is also compared with immunoreactivity for a retinal ganglion cell (RGC) marker (**F**). (**G**) The *yellow-orange* coloration in the image overlay demonstrates the presence of scGH in RGC cytoplasm and nuclei. (**H**) The specificity of detection is shown in a control NRS-treated section. *Bars,* 20μm.

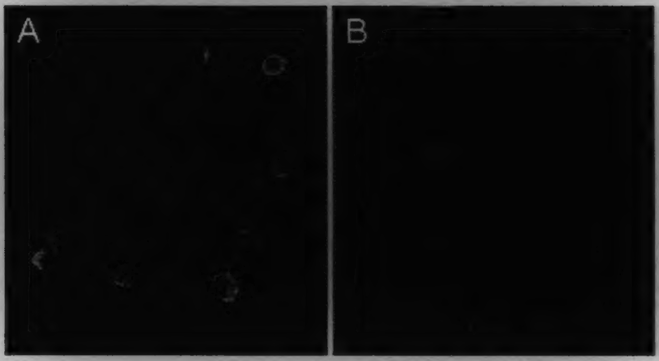

Fig. 5.11. (A) scGH immunoreactivity in the cytoplasm and nuclei of cultured Thy-1 immunopanned retinal ganglion cells (RGCs), in comparison with controls treated with normal rabbit serum (**B**).

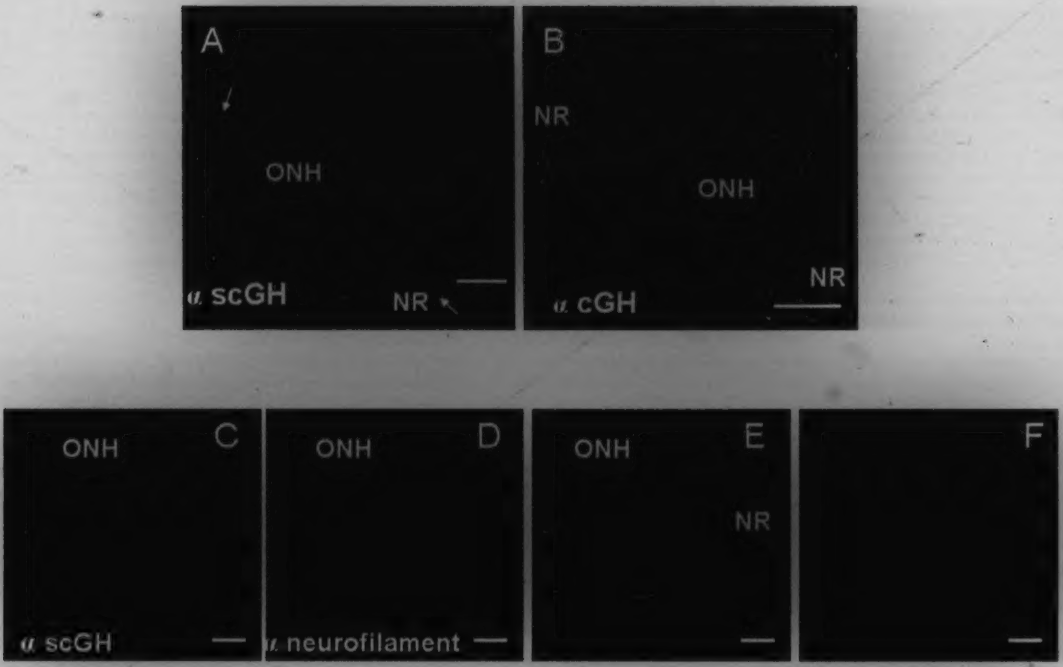
A
ONH
α scGH
NR
B
NR
ONH
NR
α cGH
ONH
C
α scGH
ONH
D
α neurofilament
ONH
E
NR
F

Fig. 5.12. (**A**) scGH immunoreactivity in the optic nerve head (ONH) and neural retina (NR) of E7 chick embryos, in comparison with immunoreactivity for native pituitary (full-length) GH (**B**). scGH immunoreactive cells are indicated by the arrows (A). (**C**) Confocal labeling of scGH immunoreactivity in the ONH and NR, in comparison with neurofilament labeling (**D**). The overlay (**E**) indicates that the neurofilament fibres have little scGH immunoreactivity. A normal rabbit serum control (**F**) is shown for comparison. *Bars,* 50μm.

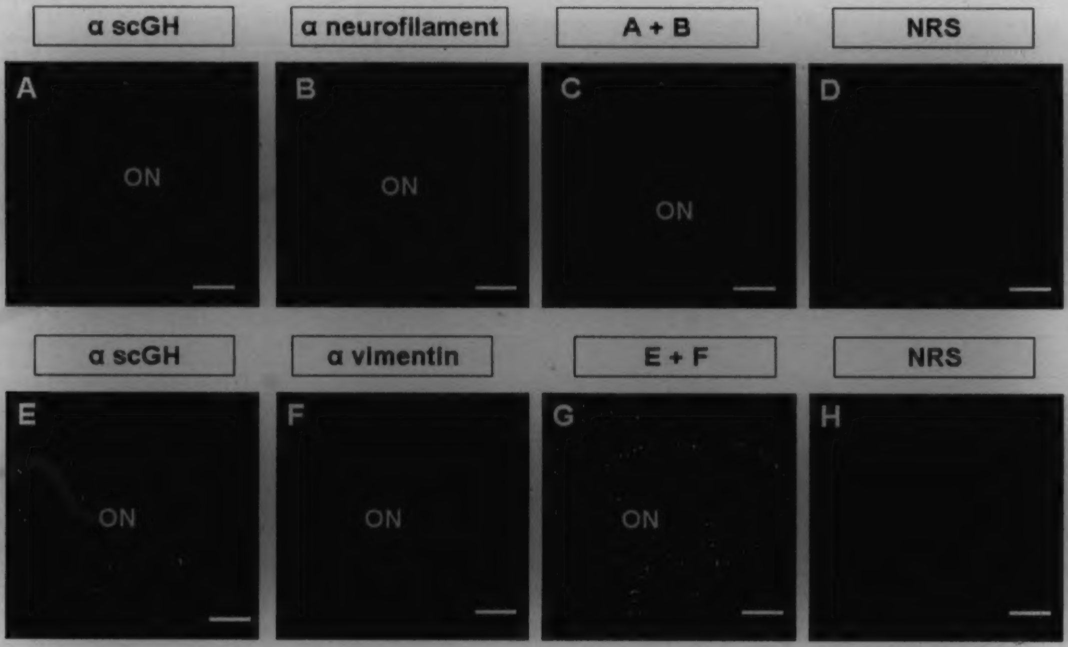
α scGH
α neurofilament
A + B
NRS
A
ON
B
ON
C
ON
D
α scGH
α vimentin
E + F
NRS
E
ON
F
ON
G
ON
H

Fig. 5. 13. (**A, E**) scGH immunoreactivity in a transverse section of the optic nerve (ON), compared with neurofilament immunoreactivity (**B**) and vimentin immunoreactivity (**F**). Confocal imaging demonstrates that most of the scGH immunoreactivity is not colocalized with neurofilament (**C**), although scGH and vimentin are colocalized (**G**). Normal rabbit serum (NRS) controls are shown for comparison (**D** and **H**). *Bars,* 50μm.

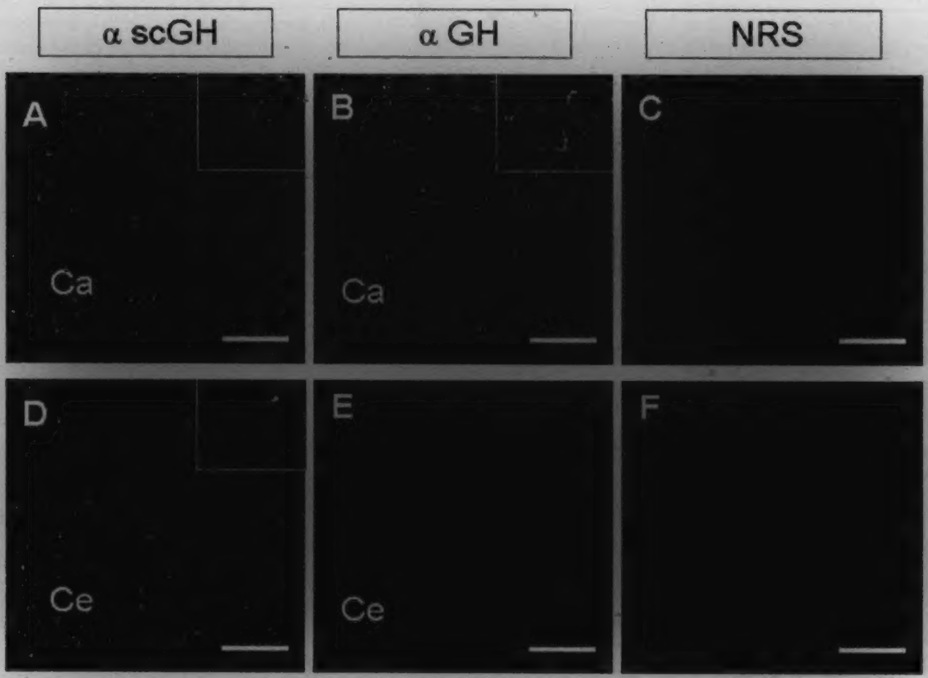

Fig. 5.14. scGH immunoreactivity in the caudal (Ca, **A**) and cephalic (Ce, **D**) lobes of the chicken pituitary gland, in comparison with immunoreactivity for native pituitary (full-length) GH in caudal (**B**) and cephalic (**E**) lobes. (**C**) and (**F**) show normal rabbit serum (NRS) controls for the caudal and cephalic lobes, respectively. *Bars,* 20µm.

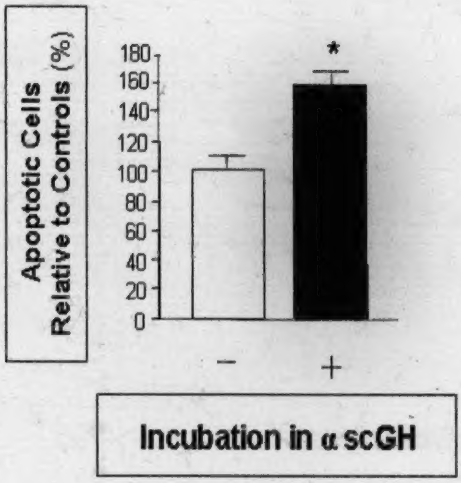

Fig. 5.15. Immunoneutralization of scGH increases cell death in immunopanned retinal ganglion cells (RGCs). RGC cultures were incubated overnight at 37 °C in the presence (*solid bar*) or absence (*open bar*) of a scGH antibody at a final dilution of 1:100. Apoptotic cells were identified by DAPI labeling. Means ± SEM (n = 9). The *asterisk* indicates that the difference between the groups is significantly different, $P<0.05$.

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Chapter 6

Small Chicken Growth Hormone (scGH) Variant in the Neural Retina: Structure-Function Correlates

Chapter 6: Small Chicken Growth Hormone (scGH) Variant in the Neural Retina: Structure- Function Correlates[1]

6.1 Introduction

A novel transcript of the growth hormone (GH) gene has been identified, by 5' RACE (Gene Bank Accession Number AB066586) in ocular tissues of embryonic chicks (Takeuchi *et al* 2001). This 569 bp transcript is transcribed from the middle of intron 3 of the full-length chicken GH gene and lacks exons 1-3. The presence of this transcript in the neural retina of embryonic chicks has since been confirmed by RT-PCR and sequencing, and it has been expressed *in vitro* in HEK 293 cells from a plasmid vector (Baudet *et al* 2005; Harvey *et al* 2006). The protein expressed by this plasmid is 16.5 kDa in size (Baudet *et al* 2005; Harvey *et al* 2006), as predicted from its primary sequence (Takeuchi *et al* 2001). It has 140 amino acid residues and lacks the signal peptide and the N-terminal 71 amino acid residues of monomer (22 kDa) chicken (c) GH, replacing them with 20 aberrant encoded by the 3' end of the intron C of the *GH* gene. The C-terminal 120 residues of scGH contain helixes 3 and 4 of monomer GH and within its primary sequence, four cysteine residues are present at similar positions as those in 22 kDa cGH, resulting in the formation of two intramolecular and / or intermolecular disulfide bridges. Takeuchi *et al* (2001) therefore suggested this small GH variant (scGH) would adopt the same secondary structure as the corresponding region of 22 kDa cGH and possess its biological and receptor-binding activities. This possibility has been assessed in the present study, by computerized modeling and structure-function analysis of the scGH variant.

6.2 Methods

Analysis of the primary structure

scGH primary structure was analyzed using PROSITE (http://www.expasy.ch/prosite/), PSORT II (Nakai and Horton, (1999);

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[1] A version of this chapter has been accepted for publication: Baudet ML and Harvey S Small chicken growth hormone (scGH) variant in neural retina: Structure-function correlates, *the Journal of Molecular Neuroscience.*

http://psort.ims.u-tokyo.ac.jp/form2.html), and predict NLS (Cokol et al., (2000); http://cubic.bioc.columbia.edu/predictNLS/) to determine scGH motifs, subcellular localization and putative nuclear localization signal (NLS), respectively.

Homology modeling

scGH sequence alignment was first performed using Clustal W (Fig. 6.1), which was then submitted to the Swiss Model Comparative Protein Modelling Server (Peitch 1995; Guex and Peitch 1997; Schwede *et al* 2003). Five templates derived from the crystal structures of human GH (the only species with a known GH crystal structure) [1HWG and 1HWH (Sundstrom *et al* 1996), 3HHR (de Vos *et al* 1992), 1HGU (Chantalat *et al* 1995), 1A22 (de Vos *et al* 1992)] were chosen as templates for the construction of a model, based on their high sequence identity with scGH (Table 6.1). Of these, 1HWG was the reference template, as it had the highest sequence identity to scGH, as its crystal structure had the highest resolution, and as it had a good R factor (Table 6.1). The final homology model of scGH was visualized using DeepView (Guex and Peitch 1997) and Pymol freewares (DeLano 2002) (http://www.pymol.org).

6.3 Results

Analysis of the primary structure

The primary structure of scGH, in common with hGH, contains 6 motifs as shown in Table 6.2, including the 'signature 2' of somatotropin, prolactin and related hormones and two leucine zipper patterns. scGH does not, however, possess a N-terminal signal peptide, an endoplasmic reticulum (ER) retention motif, peroxisome targeting signals or a transport motif for translocation from the cell surface to Golgi. It also lacks a nuclear localization signal (in common with hGH, cGH, rGH, mGH, cGHR, rGHR), although according to PSORT II, scGH is predicted to be localized to the nucleus and to the cytoplasm with a probability of 30.4% and 26.1%, respectively.

Homology modeling

scGH's tertiary structure was determined by constructing a homology model using templates derived from the crystal structure of hGH (Table 6.1), which is biologically active in chickens (Krishnam *et al* 1989; Granot *et al* 1993; Monsonego *et al* 1997). Chicken GH was not used as a template because its crystal structure is unknown.

The Swiss Model determined how well the position of each residue of the model fitted the templates using a color code. As illustrated in Figure 6.2, most of the residues of the scGH model (residues colored in green and blue) fitted the template, apart from two restricted regions (colored in red) from Ser 12 to Gln 20 and from Ser 55 to Val 58. As illustrated in Figure 6.3, the scGH model differed from hGH in structure: hGH is composed of a 4 helix bundle, characteristic of ligands for Class 1 receptors of the hematopoietic superfamily (Kossiakoff and de Vos 1999), whereas the scGH model is composed of three main helices. Helices 2, 3 and 4 of hGH (1HWG) correspond to helices 1, 2 and 3 of scGH, but they are longer by one, four and one residues, respectively (Table 6.3). Thus, the secondary structure of the C-terminal of the two molecules is similar and the variation in helix number is thus attributed to the difference in the N-terminus of the two molecules. The short N-terminus of scGH includes 20 residues derived from intron C of full-length chicken GH (Takeuchi *et al* 2001). The predicted structure of the N-terminus of scGH has no classic secondary structure (alpha-helix or beta-sheet), whereas the N-terminus of hGH is composed of helix 1 and two mini-helices located between helix 1 and 2. This difference in ribbon structure results in a difference in the overall shape of the scGH model and hGH, as illustrated in Figure 6.4.

The possibility that scGH could bind to a GHR dimer was assessed by examining the primary and hypothetical tertiary structure of scGH. hGH binds the extracellular domain (ECD) of two GHRs sequentially at its binding site 1 (or high affinity site) then at its binding site 2 (or low affinity site) (Cunningham *et al* 1991). Studies with hGH have established that only a limited number of residues contribute to the total binding energy (Kossiakoff and de Vos 1999) (Table 6.4). Sequence alignment of scGH with hGH (1HWG) demonstrates that scGH lacks 3 key residues (of 14) at site 1 and 9 residues (of 15) at site 2 (Table 6.4). Most of these "missing" residues are a direct consequence of the N-terminal truncation of scGH. At site 1, the most important residues of hGH (Fig. 6.5A) are involved in forming a hydrophobic pocket that closely docks the side chains of Trp 104 and Trp 169 (the main residues of the first receptor ECD or "ECD1") and direct intermolecular contacts are made between the most functionally important charged groups (Fig. 6.5C and E) (Clackson and Wells 1995; Clackson *et al* 1998). scGH lacks crucial residues forming this binding pocket (Fig. 6.5B), namely the homologous residue R64 of hGH, which packs directly against Trp169 (Fig. 6.5C and D), and the homologous residues 62-63 of hGH, which cover Trp 104 (Fig. 6.5E and F) (Clackson *et al* 1998). hGH Thr 60 and Pro 61 also contribute to the covering of Trp 104 (Fig. 6.5E) (Clackson *et al* 1998). Their scGH homologous residues (Leu 17 and Ser 18) are located in the vicinity of Trp 104 in this model, but the shortest distance between them and Trp 104 [5.84 Ångströms between Leu 17 (sidechain Cβ) and Trp 104 indol ring Cζ3)] would precludes any Van der Waals contact (Fig. 6.5F). No other residues were found in the vicinity of Trp 104 or Trp 169 after analysis of scGH model. As a result, it is unlikely that tight binding of ECD1 to the site 1 of scGH could occur. At site 2 (Fig. 6.6), no spatial complementarity exists between hGH and ECD2 key binding residues (Fig. 6.6A), however some contact results in the burial of Trp 104 and Trp 169 of ECD2 by hGH site 2 (Fig. 6.6C) (Bernat *et al* 2003; Walsh *et al* 2003). scGH lacks most of the site 2 residues of hGH (Fig. 6.6B) and in particular, it lacks an homologous residue for N12 and R16 (Fig. 6.6D), both of which contribute to the formation of a hydrophobic cleft for the indole ring of Trp 169 (Walsh *et al* 2003) (Fig. 6.6C). Again, no residues, other than the homologous ones, were found in the vicinity of Trp 104 and Trp 169 (Fig. 6.6D). The absence of most of the crucial binding residues suggests ECD2 is unlikely to bind to the scGH model. Overall, hGH site 1 and 2 (Fig. 6.7A) tightly bind ECD1 and 2 (Fig. 6.7C, E). Because scGH putative site 1 and 2 have a different tertiary structure (Fig. 6.7B), as compared to hGH (Fig. 6.7A), its docking to ECD1 and 2 of the GHR dimer is unlikely to occur (Fig. 6.7D, F).

6.4 Discussion

The results of this analysis provide a novel, structural model of the recently discovered small GH variant. Although two other models of scGH have been proposed previously [Q90WE4 (scGH data base accession number) http://modbase.compbio.ucsf.edu/modbase-cgi-new/index.cgi], this is the first to use a GH molecule as a template. The first model was constructed from NMR spectroscopy of human prolactin (1N9D), to which scGH shares only 22.7% sequence identity. The second was constructed from the crystal structure of ovine placental lactogen (1F6F), to which it shares only 21.6% sequence identity. Our model, based on hGH templates of known 3D structure has a much greater sequence identity (51.4% with the reference template 1HWGA) than these earlier models. However, as the 20 amino acid N-terminal of scGH is unique to this molecule, its tertiary structure (and its sequence alignment to hGH), could not be determined accurately from these hGH templates. Similarly, Thr 148 to Asp 153 of the hGH crystal structure is poorly ordered and is partly or entirely missing in most of the hGH models, including 1HWG (Kossiakoff and de Vos 1999). The 3D structure of these two regions of our scGH model is, therefore, only an approximation. The remaining residues, however, have high sequence identity with the templates (61.74% with 1HWG) and the structure derived from them should be a reasonable facsimile.

Sequence alignment of scGH with hGH shows that scGH lacks residues thought to be crucial for the binding of hGH to hGHR dimer (Cunningham *et al* 1991; Waters *et al* 2006). Indeed, according to our homology model, docking of the main ECD1 residues would be impaired at the high affinity site 1, therefore preventing the formation of a 1:1 scGH:hGHR intermediary complex. Similarly, the low affinity site 2 is virtually absent in scGH, as scGH possesses only 4 key binding residues out of 15 in hGH. Moreover, as deletion of the first 8 residues that contribute to hGH site 2 significantly impairs the dimerization of the two ECDs (Cunningham *et al* 1991), scGH is similarly unlikely to cause GHR dimerization and subsequent change in conformation of the dimer, thought to be essential for the initiation of signal transduction (Waters *et al* 2006). Taken together, this suggests that scGH could not form a 1:2 scGH:2hGHR complex and could not induce cell signaling. It is, however, possible that scGH binds to a GHR dimer in a radically different way to that induced by hGH. It may, for instance, require different residues than the one analyzed, including homologous residues to the ones buried in the site 1 interface of hGH that do not contribute significantly to its binding affinity. Moreover, scGH might bind to a GHR dimer as a homodimer, as shown for homodimeric hPrl-G129R induced Prl receptor dimerization (Langenheim *et al* 2006). Binding studies are therefore required to empirically determine if scGH binds to the chicken GHR or a related receptor. The possibility that multiple types of GHRs might exist has been suggested many times before, especially as the established biological activity of truncated GH fragments is unlikely to be mediated through the classical GHR

(Casaneuva 1992; De Palo *et al* 2006a, 2006b). Indeed, although scGH would not be able to induce cell signaling through the classical GHR, the possibility that it may have biological activity through a different receptor is supported by the fact that the immunoneutralization of endogenous scGH in purified retinal ganglion cells (RGCs) markedly increased the amount of poly (ADP-ribose) polymerase-1 (PARP-1) (Harvey *et al* 2006). This finding strongly supports the existence of a scGH-specific receptor, at least in the neural retina.

Sequence alignment of scGH with hGH also reveals that scGH is lacking a signal peptide. This is consistent with the absence of scGH immunoreactivity in the vitreous of embryonic chick eyes (Baudet *et al* 2005), in which a 15 kDa GH moiety, likely derived from the proteolysis of monomer chicken GH, is abundantly present (Baudet *et al* 2003; Sanders *et al* 2003). This possibility is also supported by the absence of scGH in culture medium of HEK cells transfected with scGH expression plasmid, although scGH is abundantly translated in these cells (Baudet *et al* 2005). It is therefore likely to be retained in scGH expressing cells in the retina (Baudet *et al* 2005) and is unlikely to be secreted from them.

The sequence of scGH also lacks any previously identified nuclear localization signal (NLS). scGH immunoreactivity has, however, been found in the nucleus of chick RGCs, using immunohistochemistry (Baudet *et al* 2005). The nuclear localization of scGH within the chick RGCs is thus surprising, although rat GH binds to nuclear compartments in rat pituitary glands (Fraser and Harvey 1992), and hypothalamic tissue (Harvey and Hull 2003), reflecting the presence of immunoreactive GHRs in the nucleus of central and peripheral tissues (Lobie *et al* 1991, 1994a, 1994b; Lincoln *et al* 1994). Indeed, monomer GH is translocated from the plasma membrane via the cytosol to the nucleus in GHR expressing cells (Lobie *et al* 1991, 1994a; Mertani *et al* 2003) and nuclear GH is present in many tissues (Rezvani *et al* 1973; Bonifacino *et al* 1983; Harvey *et al* 2000, 2001). Interestingly, the analysis of the primary structure of scGH shows two leucine zipper motifs, suggesting nuclear roles for scGH as a leucine zipper transcription factor.

In summary, we have developed a novel, structural model of scGH, with implications for its putative actions through classical GHRs.

Table 6.1. Templates used for the construction of the scGH model

#	PDB[I] ID	Chain	hGH bound to:	Resolution (Ångström)*	R-factor**	Overall % identity	% identity[']	% similarity[H]
1	1HWG	A	2hGHPB	2.50	0.199	51.35	61.74	64.86
2	3HHR	A	hGHBP:hGHR	2.80	0.221	51.35	61.74	64.86
3	1HGU	-	-	2.50	0.212	50.00	58.33	65.54
4	1HWH	A	1 hGHBP	2.90	0.215	50.68	60.87	64.19
5	1A22	A	1 GHR	2.60	0.187	47.97	56.90	61.49

The templates were all derived from the crystal structure of hGH. Note that the templates 4 and 5 have a single mutation (G120R).

[I] PDB: Protein Data Base

* Higher resolution indicates higher accuracy of atom position. To be acceptable, the resolution of the diffraction pattern of a molecule must be below 3 Ångströms.

** The R-factor is a statistical measure that tells how well the model fits the original data; a good R-factor for a model at 2.5 Ångströms resolution is 0.2.

['] % identity is calculated without the unique first N-terminal 20 residues and homologous region to 1HWGA, 2HHRA and 1HWHA's Thr148 to Asp 153, 1HGUA's Thr148, and 1A22A's Asp 149 to Asp 153 whose structures are unknown. For comparison, the % identity to the hGH primary sequence is 59.50%, excluding the 20 amino acid N-terminal.

[H] % sequence identity based on the presence of similar (rather than identical) amino acids.

References: (1) Sundstrom et al 1996, (2) De Vos et al 1992, (3) Chantalat et al 1995, (4) Sundstrom et al 1996, (5) Clackson et al 1992

Table 6.2. Motifs present in the primary structure of scGH

Motif*	**PROSITE I.D.**	**Amino Acids**	**Sequence**
Somatotropin, prolactin and related hormones signature 2	PS00338	113-130	CFKkDLhkVetYlkVmkC
leucine zipper pattern	PS00029	10-31 104-125	LhsseipLsfqdmeLlrfslvL LlknyglLscfkkdLhkvetyL
Casein kinase II phosphorylation site	PS00006	18-21	SfqD
Protein kinase C phosphorylation site	PS00005	55-57 80-80	SdR SpR
N-myristoylation site	PS00008	109-114	GLlsCF
N-glycosylation site	PS00001	137-140	NCTI

*Determined by PROSITE

Table 6.3. Comparison of the residues involved in helical structures of hGH and scGH

		From:	To:		From:	To:		From:	To:
hGH (1HWGA) helices	H2*	asn72	gln91	H3	asp107	arg 127	H4	ala 155	ser184
scGH homologous residues		asp21	gln40		asp56	glu75		ala103	arg132
scGH helices	H1	met22	gln40	H2	glu60	glu75	H3	leu 104	arg 132

* H2 = Helix 2

Table 6.4. Residues contributing to the overall binding affinity at site I (A) and site II (B)

site I hGH residues	K41	L45	P61	R64	K172	T175	F176	R178	P48	E56	Q68	R183	D171	I179
scGH homologous residues	-	**M1**	**Ser 18**	**gap**	K120	T123	Y124	K126	**C5**	**S13**	**gap**	R131	H119	V127

site II hGH residues	I4	R16	N109	F1	P2	P5	R8	L15	R19	V102	E119	N12	Y103	D116
scGH homologous residues	-	-	**gap**	-	-	-	-	-	-	V51	E67	-	F52	D64

(A) At site I, 8 major hGH residues (K41 to R178) account for 85% of the binding energy (and form the binding hot spot), whereas 6 minor residues (P48 to I179) account for the remaining 15% of the binding energy (Cunningham et al, 1991).
The scGH residues (or gap) (in bold) are located in the N-terminus.

(B) at site II, single alanine mutation of the buried residues 14 to N109, F1 to E119 and N12 to D116 induce the largest, intermediate and smallest decrease in the binding affinity to extracellular domain (ECD) 2.

- Homologous scGH residues are absent.

```
1hwhA           FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPT 60
1a22A           FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPT 60
3hhr            FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPT 60
1hwgA           FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPT 60
hGH             FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPT 60
1hgu_           -PTIPLSRLFQNAMLRAHRLHQLAFDTYEEFEEAYI---QKYSFLQAPQASLCFSESIPT 56
scGH            -------------------------------------------MQQHCRTPHLHSSEIPL 17
                                                           : *  ::.  .*..**

1hwhA           PSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEER 120
1a22A           PSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEER 120
3hhr            PSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEG 120
1hwgA           PSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEG 120
hGH             PSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEG 120
1hgu_           PSNREQAQQKSNLQLLRISLLLIQSWLEPVGFLRSVFANSLVYGASDSDVYDLLKDLEEG 116
scGH            S--------FQDMELLRFSLVLIQSWLTPVQYLSKVFTNNLVFGTSDR-VFEKLKDLEEG 68
                .         .:::***:**:****** ** :* .**:*.**:*:**  *:: ******

1hwhA           IQTLMGRLEDGSPRTGQIFKQTYSKFD------DALLKNYGLLYCFRKDMDKVETFLRIV 174
1a22A           IQTLMGRLE------GQIFKQTYSKFDT-----DALLKNYGLLYCFRKDMDKVETFLRIV 169
3hhr            IQTLMGRLEDGSPRTGQIFKQTYSKFD------DALLKNYGLLYCFRKDMDKVETFLRIV 174
1hwgA           IQTLMGRLEDGSPRTGQIFKQTYSKFD------DALLKNYGLLYCFRKDMDKVETFLRIV 174
hGH             IQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIV 180
1hgu_           IQTLMGRLEDGSPRTGQAFKQTYAKFDN-SHNDDALLKNYGLLYCFRKDMDKVETFLRIV 175
scGH            IQALMRELEDRSPRGPQLLRPTYDKFDIHLRNEDALLKNYGLLSCFKKDLHKVETYLKVM 128
                **:** .**. :.   * :: ** ***    ..********** **:**:.****:*:::

1hwhA           QCRSV-EGSCGF 185
1a22A           QCRSV-EGSCGF 180
3hhr            QCRSV-EGSCG- 184
1hwgA           QCRSV-EGSCG- 184
hGH             QCRSV-EGSCG- 190
1hgu_           QCRSV-EGSCG- 185
scGH            KCRRFGESNCTI 140
                :** ..*..*
```

Fig. 6.1. Multiple sequence alignment of scGH to the five Protein Data Base templates and to hGH used to build the homology model. hGH primary sequence is the only complete sequence and was therefore included in the sequence alignment. I140 was omitted by Swiss Model during the homology modeling process.

Fig. 6.2. Illustration of how well the residues of the scGH model fitted the hGH template. Residues whose position was determined by Swiss Model with a high degree of confidence, because it fitted the template, was colored towards the blue end of the spectrum, whereas residues whose position was determined with a lower confidence were colored in red.

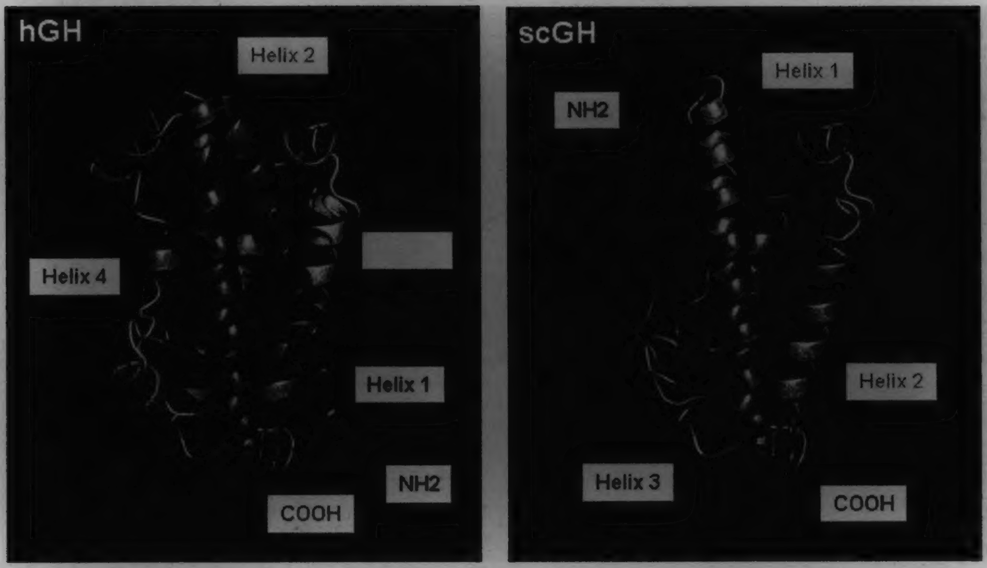
hGH
Helix 2
Helix 4
Helix 1
NH2
COOH
scGH
Helix 1
NH2
Helix 2
Helix 3
COOH

Fig. 6.3. Representation of the hGH (1HWG) and scGH model. The position of each secondary structure is indicated by colors of the visible spectrum (blue is N-terminal and red is C-terminal). Note the different structure and length of the N-terminus between the two molecules.

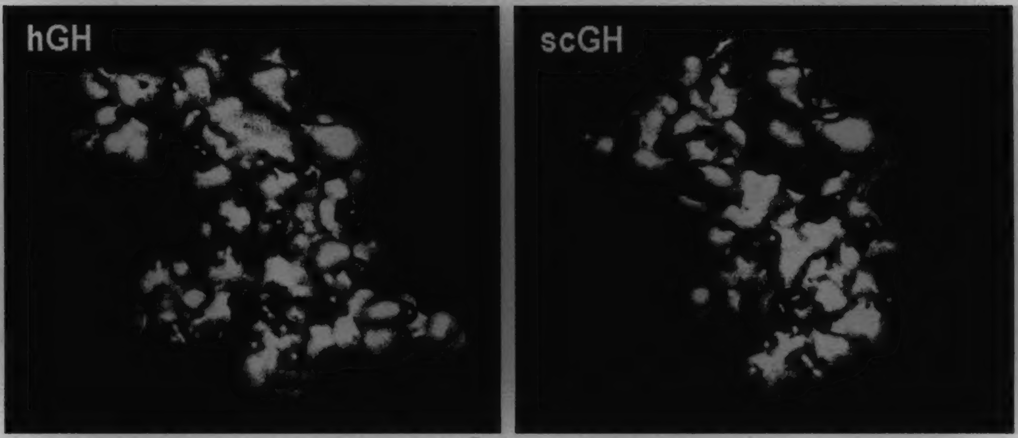

Fig. 6.4. Representation of the Van der Waals surface of the hGH (1HWG) and scGH models in CPK colors (the carbon is colored white, oxygen red, hydrogen white, sulfur yellow, sodium blue). Note the overall different shape of the molecules.

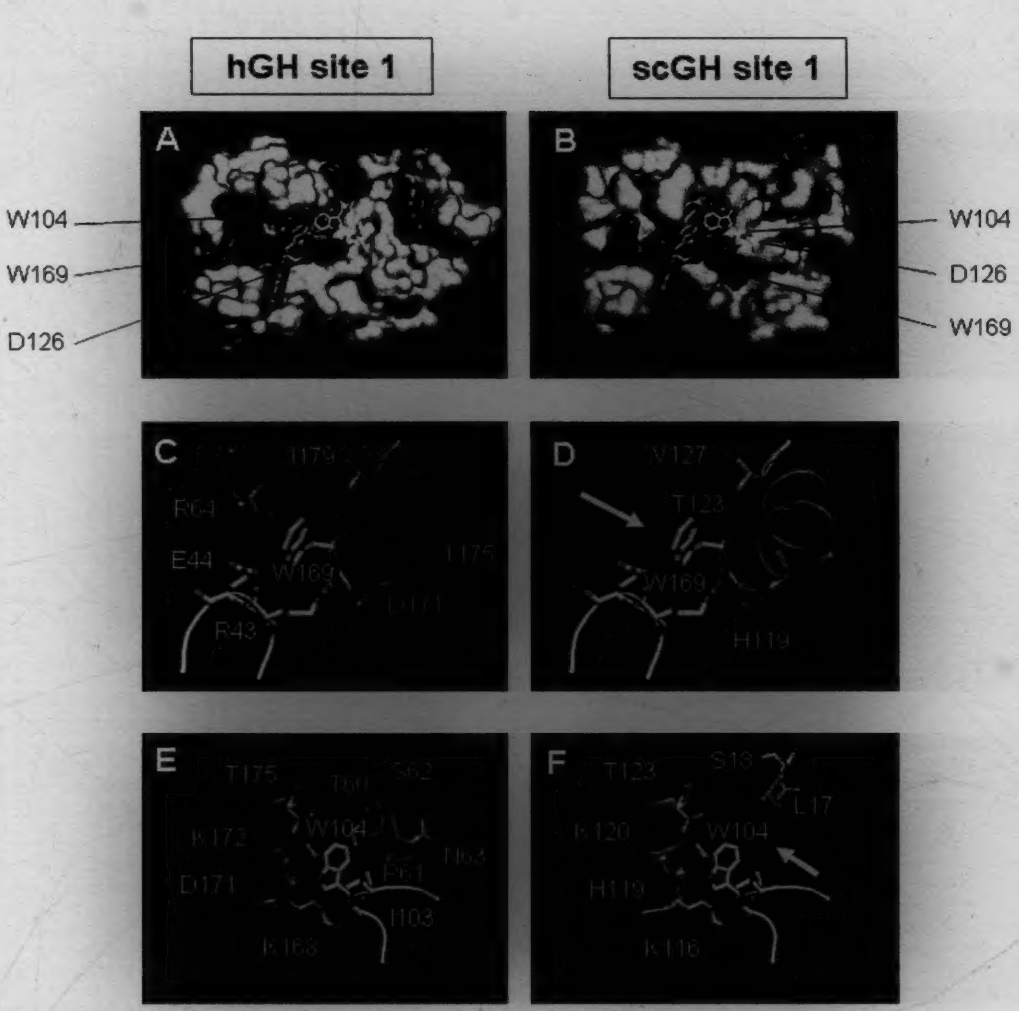
hGH site 1
scGH site 1
A
W104
W169
D126
B
W104
D126
W169
C
I179
R64
E44
W169
T175
D171
R43
D
V127
T123
W169
H119
E
T175
T60
S62
K172
W104
N63
D171
P61
I103
K168
F
T123
S18
L17
K120
W104
H119
K116

Fig. 6.5. Topographical distribution of hGH (1HWG) residues accounting for the total binding affinity at site 1, and their scGH homologues. Representation of the Van der Waals surface of hGH (1HWG) (**A**) and of the scGH model (**B**). Major and minor residues, contributing to the binding affinity at site 1, are colored in red and blue, respectively (A). The homologous residues of scGH are represented with the same color-code (B). The important residues of the extracellular domain (ECD) 1 are colored in yellow (modified from Walsh et al 2003). Illustration of the hydrophobic pocket formed by the side chains of hGH key residues at the site 1 (**C** and **E**) interface (modified from Walsh et al 2003). **D** and **F** represent the homologous residues of scGH. Note the absence of key residues (arrow) in the scGH model.

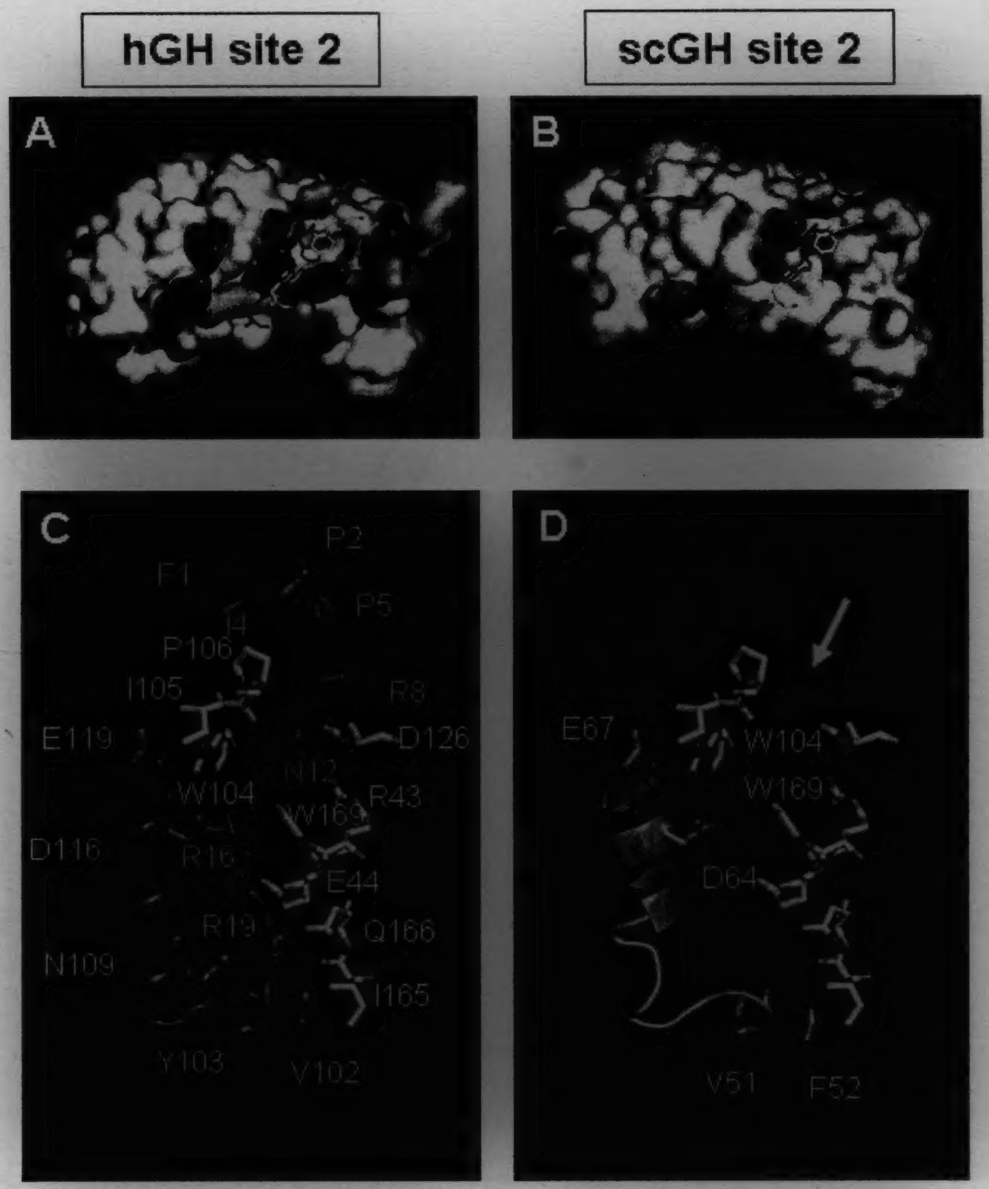
hGH site 2
scGH site 2
A
B
C
P2
F1
P5
I4
P106
I105
R8
E119
D126
N12
W104
R43
W169
D116
R16
E44
R19
Q166
N109
I165
Y103
V102
D
E67
W104
W169
D64
V51
F52

Fig. 6.6. Topographical distribution of hGH (1HWG) residues accounting for the total binding affinity at site 2 and their scGH homologues. Representation of the Van der Waals surface of hGH (1HWG) (**A**) and of the scGH model (**B**). The side chain accounting for the binding energy at site 2 are also represented (the residues that show the largest, intermediate and smallest effects on binding affinity upon mutation into alanine are colored red, cyan and blue, respectively) (A). The homologous residues of scGH are represented with the same color-code (B). The important residues of the extracellular domain (ECD) 2 are colored in yellow (modified from Walsh et al 2003). (**C**) Illustration of the hydrophobic pocket formed by the side chains of hGH key residues at the site 2 interface (modified by Walsh et al 2003). (**D**) represents the homologous residues of scGH. Note the absence of key residues (arrow) in the scGH model.

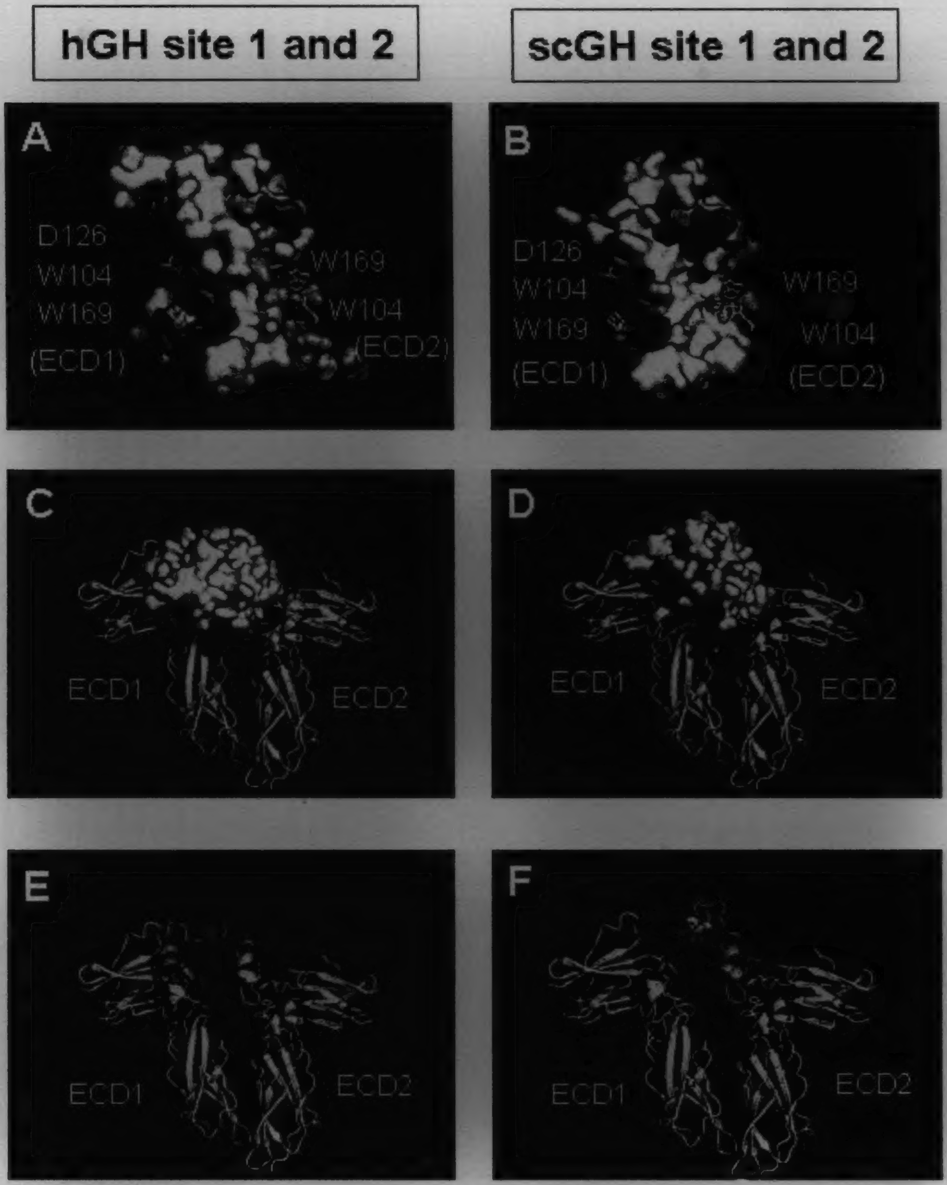
hGH site 1 and 2
scGH site 1 and 2
A
D126
W104
W169
(ECD1)
W169
W104
(ECD2)
B
D126
W104
W169
(ECD1)
W169
W104
(ECD2)
C
ECD1
ECD2
D
ECD1
ECD2
E
ECD1
ECD2
F
ECD1
ECD2

Fig. 6.7. Representation of the Van der Waals surface of hGH (1HWG) and scGH that is free (**A** and **B**) or bound (**C** and **D**) to first (ECD1) and second (ECD2) extracellular domain of the GHR. The key binding residues of hGH and their scGH homologues are represented using the same color code as the ones used in Figure 6.5 and 6.6. The key GHR binding residues are in yellow. C and D is a "superior" view of hGH and scGH in A and B. Ribbon representation of hGH (1HWG) (**E**) and scGH (**F**) bound to ECD1 and 2.

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De Palo E.F., Gatti R., Antonelli G., and Spinella P. (2006) Growth hormone isoforms, segments/fragments, Does a link exist with multifunctionality? *Clin. Chim. Acta* **364**, 77-81.

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Chapter 7

Growth Hormone Actions in the Developing Neural Retina: a Proteomic Analysis

Chapter 7: Growth Hormone Actions in the Developing Neural Retina: a Proteomic Analysis[1]

7.1 Introduction

It is now well established that, postnatally, GH is primarily produced by somatotrophs within the anterior pituitary and acts on distant target sites, including skeletal, metabolic, reproductive, immune and nervous tissues [1]. These tissues are not, however, just sites of GH action, since they have all been found to be extrapituitary sites of GH production [2]. For instance, GH has been detected in the peripheral (PNS) and central nervous system (CNS) in embryonic chicks [3-5] and in the CNS of fetal and neonatal rats [6, 7] and the human fetus [8], suggesting roles for GH in neural development. This possibility is supported by the presence of the GH receptor (GHR) / GH binding protein (GHBP) in the CNS and PNS of early chick embryos [3, 4], fetal and neonatal rodents [6, 9-13], neonatal rabbits [12, 13], fetal bovines [14] and fetal humans [15, 16]. Exogenous GH has also been shown to promote cell proliferation and cell differentiation in cultures of fetal rat cortical cells in neonate mice subventricular stem cells and in fetal neuroepithelial cells [6, 17], although GH roles in developing nervous tissues are still largely unknown.

Within the CNS, the developing neural retina has recently been established as an important site of GH synthesis and action. In chicks, GH is synthesized and produced by retinal ganglion cells (RGCs) within the embryonic day (E) 7 neural retina [18]. GH is present within the cell soma of these cells but can also be traced within their axons in the retina within the optic fibre layer and outside the retina within the optic nerve, optic chiasm and optic tract [19]. GHR is synthesized in the neural retina at a similar developmental period [20] and GH has recently been found to prevent cell death *in vitro* and *in vivo* in the neural retina between E6-8 [21-23]. This likely reflects an autocrine /

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[1] A version of this chapter will be submitted to *Proteomics* for publication: Baudet ML, Hassanali Z, Sawicki G, List E, Kopchick JJ, and Harvey S Growth hormone actions in the developing neural retina: a proteomic analysis. E List and JJ Kopchick provided the KO mice and performed the genotyping, G Sawicki assisted with the 2-DE gel electrophoresis experiments, Z Hassalani assisted with the *in situ* hybridization.

paracrine mode of action of endogenous GH, since its immunoneutralization induces an increase in retinal cell death in cell culture of purified E8 RGCs [21].

The rat neural retina is also a site of GH production, as GH has been detected within this tissue in neonatal animals [24]. Similar to the studies in chick embryos, GH mRNA and protein are mostly found in the ganglion cell layer (GCL) and GH protein is also present in the optic fibre layer in newborn rats [24]. GHR / GHBP has similarly been detected within all cells of the neural retina in E12-18 fetal rats [11] and in postnatal day (P) 10 rats, in which they appear to be particularly abundant in the GCL [12], suggesting it is a putative GH target site. Roles for GH within the rodent retina are, however, unknown and the focus of this study, using newborn GHR knockout mice and a proteomic approach.

7.2 Materials and methods

Animals

The newborn (P0) mice used in these studies were housed in the transgenic facility at the Edison Biotechnology Institute and have been described previously [25]. GHR (-/-) mice and their controls (+/+) were obtained from the inbreeding of GHR (+/-) mice. Genotypes were confirmed by RT-PCR of total RNA from the tails of mice collected at autopsy [26]. Animals were housed in a room with a controlled photoperiod of 12-h light:12-h darkness (lights on from 06:00 to 18:00 h) and a temperature 22–37 °C. Mice were given free access to a nutritionally balanced diet (Lab Diet: PM1 Feeds, St. Louis, MO, USA) and tap water. All experiments were approved by the University Animal Care and Utilization Committee, and were conducted in accordance with NIH Animal Care guidelines. The possibility that the mouse neural retina might be a site of GH production was assessed using eyes of newborn balb/C mice (Charles River Wilmington, MA, USA) and the collection of tissues from these animals was with approval of the University of Alberta's Animal Welfare Committee.

***In Situ* Hybridization for GH mRNA**

Cloning. To synthesize GH riboprobes, a 693 bp full-length rat pituitary GH cDNA obtained by using the primers JAB1 and JAB2 was subcloned into the PCR II-TOPO vector (Invitrogen), as detailed previously [18, 27]. The analyzed sequence of the insert was verified by the University of Alberta DNA Core Facility.

Probe synthesis. The purified vectors were linearized using the restriction endonucleases NotI and BamHI for the subsequent production of sense and antisense probes, respectively. Digoxigenin (DIG)-labeled antisense and sense riboprobes were synthesized by *in vitro* transcription with T7 and SP6 RNA polymerase, respectively, and DIG RNA labeling mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Probe concentration was determined by dot blot analysis against a DIG-labeled RNA standard.

Hybridization procedure. The eyes of newborn mice and the pituitaries of adult mice (as a positive control) were fixed overnight at 4 °C in 4% paraformaldehyde (PFA) in PBS, rinsed in PBS (2 x 5 min), and dehydrated in a gradient series of alcohol (50%, 70%, 95%, 100% ethanol), cleared with Citri-solv (Fisher, Edmonton, AB, Canada) and embedded in paraffin wax overnight at 60 °C under normal atmospheric pressure. 6 μm tissue sections were cut using a microtome, mounted onto charged slides (Fisher) and hydridization was performed as previously described [24]. In brief, the tissue sections were deparaffinized in Citri-solv, rehydrated in a gradient series of alcohol, washed in diethylpyrocarbonate (DEPC)-treated water and then in DEPC-PBS. The sections were then incubated in fresh 0.1% DEPC-PBS (2 x 15 min) to remove RNase, treated with proteinase K (10 μg/ml, 10 min, 37 °C), postfixed in 4% PFA (10 min), and incubated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. The sections were then prehybridized with 50% formamide / 2x saline sodium citrate (SSC) (30 min, 60°C) and hybridized in a moist chamber (overnight, 60 °C) with hybridization solution (50% formamide, 2x SSC, 250 μg/ml tRNA, 50 μg/ml heparin, 2% blocking solution), and denatured (5 min at 80 °C) sense or antisense DIG-labeled riboprobes at concentrations of 0.4 to 2 μg/ml. The sections were sequentially washed in 2x SSC (10 min), 2x SSC / 50% formamide (45 min, 45 °C), 2x SSC (3 x 15 min), 2x SSC (30 min, 37 °C), 0.1x SSC (60 min, 60 °C), and then washed (2 x 5 min) in buffer one (0.1 M Tris, 0.15 M NaCl, pH 7.5). Immunohistochemical detection of the hybridized probe was achieved by incubation (2h) with an anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics) in 1% blocking solution (Roche Diagnostics). The sections were then washed in buffer one (2 x 15 min), equilibrated (5 min) in buffer two (0.1 M Tris, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5), and incubated in buffer two containing a commercial substrate for alkaline phosphatase [5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium chloride; Roche Diagnostics] until color developed. Color development was stopped by washing in Tris-EDTA buffer (2 x 5 min). The sections were then rinsed in PBS (3 x 5 min) and mounted with an aqueous medium (Crystal / Mount, Biomeda, Foster City, CA, USA).

Immunohistochemistry

Fixation. Whole eyes were collected and placed in Z-fix (Anatech Ltd, Battle Creek, MI) at 4 °C until further processing. They were then washed (2 x 15 min) in PBS (pH 7.4) and dehydrated in a graded series of ethanol (50%, 30 min; 70%, 30 min; 95%, 30 min; 100%, 2 x 30 min) and cleared with Citri-solv (Fisher) for 30 min. Tissues were then infiltrated with paraffin wax at 60 °C for 3 hours under vacuum. Serial transverse tissue sections (6 μm) were taken using a microtome and mounted onto charged slides (Fisher).

Confocal microscopy. Sections were cleared in Citrisolv (2 x 5 min), rehydrated in a graded series of ethanol (100%, 2 x 5 min; 95%, 2 min; 70%, 2 min; 50%, 2 min; distilled water, 2 min), equilibrated in PBS (2 min). The sections were then blocked with 10% NGS (in PBS containing 0.4% tween) for 1 h and incubated overnight at 4 °C with primary antibody. This specific antibody was raised in rabbit against the amino terminus of human GH (Santa Cruz Biotechnology, Santa Cruz, CA) and has been shown by the manufacturer to cross-react with both rat and mouse GH (diluted 1:250). After incubation with the primary antibodies, the sections were incubated for 1 h at room temperature in goat anti-rabbit IgG conjugated to Alexa Fluor 555 ($F(ab')_2$ fragment, 4 μg/ml, Molecular Probes). The sections were then washed (3 x 10 min) and mounted with Gel mount (Biomeda, Foster City, CA). The labeled sections were examined using a LSM 510 confocal microscope (Carl Zeiss, Göttingen, Germany), equipped with appropriate lasers, using an optical slice of one airy unit. Digital images were brightness and contrast balanced.

Proteomic analysis

2-D electrophoresis (DE). GHR (-/-) ($n = 6$) and GHR (+/+) ($n = 6$) mice were killed on the day of their birth. Neural retinas were excised and flash frozen in liquid nitrogen for

2-DE gel analysis. Protein samples for 2-DE were prepared by mixing the frozen retinas with rehydration buffer (8 M urea, 2% CHAPS, 10 mM DTT, 0.2% Bio-Lytes 3/10 ampholyte, 0.001% Bromophenol Blue, Bio-Rad, Mississauga, Ontario, Canada), which was sonicated (3x, 0.5 s) on ice and centrifuged for 2 min at 15,000 rpm at room temperature, to remove any insoluble particles. The protein content of retinal samples in rehydration buffer was measured using the Bio-Rad protein assay after suitable dilution of the samples, and 75 μg protein was applied to 11-cm IPG (Immobilized pH Gradient) strips, which had a linear pH gradient from 3–10 (Bio-Rad). For isoeletric focusing (IEF), the Bio-Rad protean IEF cell was used at 20 °C with fast voltage ramping: step 1: 15 min with an end voltage of 250 V; step 2: 150 min with an end voltage of 8000 V; step 3: 35,000 Vh . After IEF, the strips were equilibrated according to the Bio-Rad protocol. The second dimension of 2-DE was carried out using Criterion precast gels (8–16%) (Bio-Rad) in a Criterion Dodeca Cell (Bio-Rad). After separation, the proteins were detected using mass spec compatible silver staining (Silver Stain Plus, Bio-Rad) due to its high sensitivity. Since not all proteins can be detected with silver staining, the gels were destained with 1% hydrogen peroxide according to bio-rad protocol and subsequently restained with coomassie brilliant blue R-250 (Bio-Rad) but no additional spots were detected. To minimize variation in staining, all gels were stained in the same bath. The reproducibility of protein resolution using this 2-DE technique and staining procedure is very high, as detailed previously [28, 29]. In our laboratory, the intensity of staining for a single protein when run on separate gels differs by <5% and the correlation coefficient (r^2) for the staining intensities of multiple protein samples measured on different gels is > 0.9 [28]. This procedure therefore obviates the need for replicate determinations, as previously established [28-32].

Image analysis. Developed gels were scanned using a GS-800 calibrated densitometer (Bio-Rad). Quantitative analysis of spot intensity was performed using PDQuest 7.1 software (Bio-Rad). The protein spot sensitivity threshold was used to determine significant changes in protein spot size and intensity. This was based on four parameters: minimum peak value sensitivity, smallest spot area, largest spot area and the noise filter level. Only spots with relative intensities between 6 and 100 arbitrary units were considered for MS analysis.

Mass spectrometry (MS). Electrophoretically separated proteins from the spots that demonstrated statistically significant changes in intensity (at the $p < 0.05$ level) were excised from the gels and separately pooled. In-gel digestion was performed on a MassPrep Station (Micromass, Manchester, UK), using the method supplied by the manufacturer. Excised gel pieces were destained, reduced with DTT and the cysteine residues reacted with iodoacetamide, before digestion with trypsin (Promega sequencing grade) and extraction. The extract was then analyzed via LC-MS / MS. LC was performed on a Waters CapLC (Milford, MA, USA), using a water / ACN (0.2% formic acid) gradient, on a PicoFrit Capillary column (New Objectives, Woburn, MA, USA) (BioBasic C18, 5-mm particle size, 10 cm 675 mm id, 15-mm tip). The eluted peptides were then electrosprayed and analyzed on a MicroMass Q-TOF 2, using automated data-dependent MS to MS / MS switching [29].

The resultant MS / MS data were searched against NCBInr and Swiss-Prot databases for identification of the protein. A mass deviation of 0.6 was tolerated and 1 missed cleavage sites were allowed in the searches. The MASCOT (www.matrixscience.com) search engine was used to search the NCBInr protein database for protein identification. The MOWSE scoring algorithm was used for justification of accuracy of protein identification and is incorporated in the MASCOT search engine [33].

7.3 Results

To assess the possibility that the mouse neural retina might be an autocrine or paracrine site of GH production, the possible localization of GH mRNA and protein in this tissue was first investigated.

GH mRNA in the neural retina

Using *in situ* hybridization, an antisense probe designed to target the full length GH mRNA hybridized with adult mouse pituitary tissue (Fig. 7.1A). A strong signal was detected within the cytoplasm of pituitary cells, although some cells remained unstained (Fig. 7.1A inset). For comparison, no signal was found when the sense probe was used (Fig. 7.1B). This staining pattern suggests that the antisense riboprobe was specific for GH mRNA.

A signal detected with GH antisense probe was also found within the neural retina of newborn mice (Fig. 7.1C). Its intensity was particularly strong within the RGC layer (GCL) (Fig. 7.1E-F) and it was also present, albeit to a lesser extent, in cells of the developing inner nuclear layer adjacent to the inner plexiform layer (Fig. 7.E-F). GH mRNA was also detected in extraretinal ocular tissues in the lens (Fig. 7.1C, E) and in vitreal blood vessels (Fig. 7.1E,G). No signal was found when the sense probe was used (Fig. 7.1D).

GH protein in the neural retina

Using immunohistochemistry, GH was detected within the cytoplasm of numerous cells in adult rat pituitary tissue (Fig. 7.2A), indicating that the antibody was specific for rodent GH.

Within the newborn mouse eye, a positive signal was found throughout the neural retina; it was detected within the cytoplasm of cells in the neuroblastic layer (Fig. 7.2C), composed of proliferating and newly postmitotic cells [34, 35], and appeared particularly intense in the GCL (Fig. 7.2C), within the cytoplasm of cells morphologically resembling RGCs but also in neurites emanating from these cells (Fig. 7.2D). In addition, the signal was strong in the acellular inner plexiform layer, composed of neurites from the RGCs and amacrine cells at this stage [36] and it was also present in the optic fibre layer (Fig. 7.2C-D). In contrast, no staining was detected when the primary antibody was replaced by normal serum at similar dilution (Fig. 7.2B).

GH roles in the developing newborn mouse retina

To screen for putative GH roles, proteins extracted from the retinas of P0 GHR knockout mice and wild type mice were subjected to proteomic analysis. Electrophoretic (2-DE gel) analysis identified approximately 370 protein spots per gel, of which 10 spots (approximately 3 %) differed significantly in protein content at the $p < 0.05$ (Student t-test and Mann-Whitney Signed-Rank test) level [9 were of higher abundance in the GHR (–/–) group, 1 was of lower abundance]. These spots were excised from the gels for MS analysis. Only 4 from 10 had enough protein for definitive MS identification. The protein identity, accuracy of protein identification (MOWSE score), and the number of matched peptides for these proteins are summarized in Table 7.1.

Mean spot intensities of proteins from GHR (+/+) and GHR (-/-) neural retinas are displayed for protein kinase C (PKC) inhibitor 1 [Fig. 7.3A-B, retinal content increased by 74.0 % in the GHR (-/-) group], cyclophilin A (cyclosporine A-binding protein) (Fig. 7.3C-D, retinal content increased by 190.8% in the GHR KO group), signal transduction-associated protein 3 (Fig. 7.3E-F, retinal content increased by 93.8% in the GHR transgenic group), and brain abundant, membrane attached signal protein-1 (BASP-1) (Fig. 7.3G-H, retinal content decreased by 57.1% in the GHR KO group).

7.4 Discussion

The results of this study demonstrate that GH is present and expressed in the mouse neural retina. They also establish, for the first time, that deficient GH signaling in this tissue results in downregulation of BASP-1 and the upregulation of PKC inhibitor-1, cyclophilin A, and signal transduction associated protein-3. This, therefore, suggests that GH is normally bioactive in the mouse neural retina and normally upregulates BASP-1 and downregulates PKC inhibitor-1, cyclophilin A and signal transduction associated protein-3.

GH distribution within the developing rodent retina

GH protein and mRNA have previously been found in the developing neural retina of newborn rats and in chick embryos [3, 18, 24]. In rats, GH protein and mRNA were similarly detected in cells of the GCL, in the optic fiber layer and in the developing inner nuclear layer [24]. GH protein was, however, mostly detected in cell nuclei of the GCL and was not detected in the inner plexiform layer [24]. Similarly, in E7-9 embryonic chicks, GH mRNA and protein were mostly detected in RGCs, and GH protein was particularly abundant in the nucleus of RGCs and in their axons [18, 19], although it was also found in the cells of the inner nuclear layer adjacent to the inner plexiform layer [37]. The distribution of GH mRNA is thus identical in all species studied so far but the distribution of GH protein differs slightly. Indeed GH immunoreactivity is found in the neuroblastic layer in mice, contrary to that observed in rat and chicken. In addition, it is not detected in the nuclei of cells in the GCL in mice, while nuclear staining is observed in rat and chicken [18, 24]. These differences are likely to reflect the different fixative used in this study (Z-fix as opposed to PFA), especially as the distribution of GH protein in the neural retinas of PFA fixed newborn mice is identical to that of rats and chicks (data not shown).

Neural GH immunoreactivity likely originates from cells of the GCL and the developing inner nuclear layer, since GH mRNA was found within these layers. As GHR immunoreactivity has also been found in the neural retina of fetal and neonatal rodents [11, 12], GH may act as a retinal autocrine / paracrine. Extraretinal GH could also act on retinal GHR. GH could thus come from developing retinal and vitreal blood vessels, where GH protein and mRNA are also detected at this stage. GH could also be derived from the circulation, since the pituitary gland secretes GH at this stage [38] and since systemic GH could cross the blood ocular barrier. Indeed although the barrier is not permeable to ^{125}I-bGH in adult bovines [39], it is leaky perinatally [40] and correlated decreases in vitreal and serum GH levels occur neonatally in rats [41].

GH roles within the developing rodent retina

GH is found in the mouse newborn neural retina, when cell differentiation, cell proliferation, cell migration, neurite outgrowth and retinal vascularization occur [34-36]. GH could thus be involved in any of these processes. To determine the identity of molecules mediating GH action within the neural retina, and thus putative GH roles, the proteome of GHR knockout mice was compared to that of wild type animals. A similar approach has previously been used to study GH roles in the developing rodent lung [27].

Using 2-DE gel electrophoresis, 3% of the newborn retinal proteome content was found to differ in GHR knockout as compared to wild type mice. This suggests that GH has hitherto unsuspected roles of possible physiological importance within the developing neural retina. GHR knockout animals displayed higher levels of PKC inhibitor 1, cyclophilin A, and signal transduction associated protein 3, and lower levels of BASP-1, indicating that the synthesis of these proteins is normally regulated by GH stimulation. To the best of our knowledge, this is the first report of GH regulation of these proteins, indicating GH might employ these molecules to mediate retinal specific actions.

In the absence of GHR mediated signaling, PKC inhibitor-1 levels increased, indicating that GH stimulation induces a decrease in this molecule in the wildtype neural retina. PKC inhibition has been shown to prevent cell proliferation, neurite outgrowth and angiogenesis in the retina [42-44]. An inhibition of PKC signaling abolishes the ATP-induced proliferation of E6-8 chick embryo retinal cells in culture [44]. Exogenous GH has also been shown to promote cell proliferation in adult fish retina [45], possibly by acting directly on retinal cells which express GHRs [46] and could thus similarly promote retinal cell proliferation in wildtype mice by suppressing PKC inhibitor-1 levels. Retinal vascularization is initiated in newborn mice by the development of the superficial vascular plexus [47] and PKC inhibition has been shown to prevent the growth of retinal microvascular endothelial cells induced by hepathocyte growth factor in bovines [43]. The possible involvement of GH in the ontogeny of retinal vascularization is suggested by the reduced number of retinal vascular branching points observed in patients suffering from Laron dwarfism [48], characterized by a GHR defect [49]. Conversely, high levels of GH are associated with retinopathy of prematurity, a disease characterized by abnormal growth of retinal vessels [50]. It is thus possible that GH induces retinal angiogenesis through a suppression of PKC inhibitor-1 expression and this effect might be induced by locally produced GH, as GH is found within the retinal blood vessels of the superficial vascular plexus, as shown here. PKC inhibitor activity reduces laminin-induced axon outgrowth of amacrine cells in neonatal rat neural retina cells *in vitro* [42]. Although GH has not been involved in axon outgrowth, it has been suggested to increase neurite length and number in fetal rat cortical cell culture [17], and to increase penile fibre growth and to induce sciatic nerve regeneration in adult rats [51-53]. This suggests that GH signaling might induce axon growth in the retina via PKC inhibitor-1 downregulation.

Cyclophilin A, the cytosolic isoform of the cyclophilin family of proteins, levels were reduced in GHR knockout animals, indicating that GH stimulation induces a decrease in this molecule in the neural retina of newborn wildtype mice. Although cyclophilin A, has not been previously detected in the neonatal mouse retina, it has been correlated with a loss of retinal function in mice [54, 55] and its expression in the visual cortex has been reported to be reduced following retinal lesions [56]. Exogenous cyclophilin A promotes proliferation, migration, invasive capacity, and tubulogenesis of endothelial cells at low concentrations, whereas at high concentrations it decreases endothelial cell migration and viability [58]. GH signaling might thus mediate retinal vascularization also, by preserving low levels of cyclophilin A in the neural retina of newborn wildtype mice.

Signal transduction associated protein 3 was upregulated in GHR knockout, indicating that GH stimulation induces a decrease in this molecule in the neural retina of wildtype mice. KH domain-containing, RNA-binding, signal transduction-associated protein 3 [also referred to as Sam68-like mammalian protein 2 (SLM-2) and RNA-binding protein Etoile/T-Star] was recently discovered [59, 60] and belongs to the STAR (signal transduction and activation of RNA) family of proteins [61]. SLM-2 has been shown to regulate the alternative splicing of VEGF and of tau [62-65], two proteins that have been involved in the ontogeny of the superficial retinal vasculature and of axon growth, respectively [47, 66] and that are found in the developing retina [47, 67]. It is thus possible that GH regulates the expression of various isoforms of VEGF and tau via the downregulation of SLM-2 and thus indirectly regulates retinal vascularization and axon growth.

Finally, the neural retina of GHR knockout mice had reduced levels of BASP-1, suggesting that it is likely to be a GH-response gene. BASP-1 is a member of a group of brain soluble proteins (BASPs) [68, 69] that are present in the mouse neural retina from embryonic day 13.5 of development [70, 71], are amongst the most abundant genes expressed in retinal ganglion cells [72] and are well established axon growth promoters and neuronal pathfinding modulators [69]. BASP-1 (also referred to as CAP-23 and NAP-22) has been involved in nerve regeneration, sprouting and synaptic growth [73-76]. The fact that GH signaling upregulates BASP-1 suggests a role for GH in axon elongation, nerve branching and synaptogenesis. It is thus of interest that this change in BASP-1 levels mediated by GH occurs at stages of RGC ispilateral axon elongation along their targets (at E18-P0) (the superior colliculus and lateral geniculate nucleus) [77], and at a stage when RGC axons send collaterals and branches within the superior colliculus (P0-3) [78].

GH might thus exert various roles within developing retinal cells of normal newborn mice, including retinal vascularization (via PKC inhibitor-1 and cyclophilin A and possibly SLM-2 downregulation), retinal cell proliferation (via PKC inhibitor-1 downregulation), neurite outgrowth / branching (via PKC inhibitor-1 and possibly SLM-2 downregulation, and BASP-1 upregulation) and synaptogenesis (via BASP-1 upregulation). Since GH is expressed and present in the retina, GH might act directly on retinal cells in an autocrine / paracrine manner.

Table 7.1. Identification of retinal proteins

Spot #	Probably based MOWSE Score [a]		Peptide matched	Sequence coverage (%)	***Protein identity***
	Threshold ($p<0.05$)	Observed score			
1	26	41	1	5	Protein kinase C inhibitor 1
2	28	108	5	22	Cyclophilin A (cyclosporin A-binding protein)
3	28	57	1	2	Signal transduction-associated protein 3
4	35	397	11	43	Brain abundant, membrane attached signal protein-1

a) -10Xlog(P) where P is the probability that the observed match is a random event.
All detected spot had a max missed cleavages of 1 and a peptide mass tolerance of 0.6Da

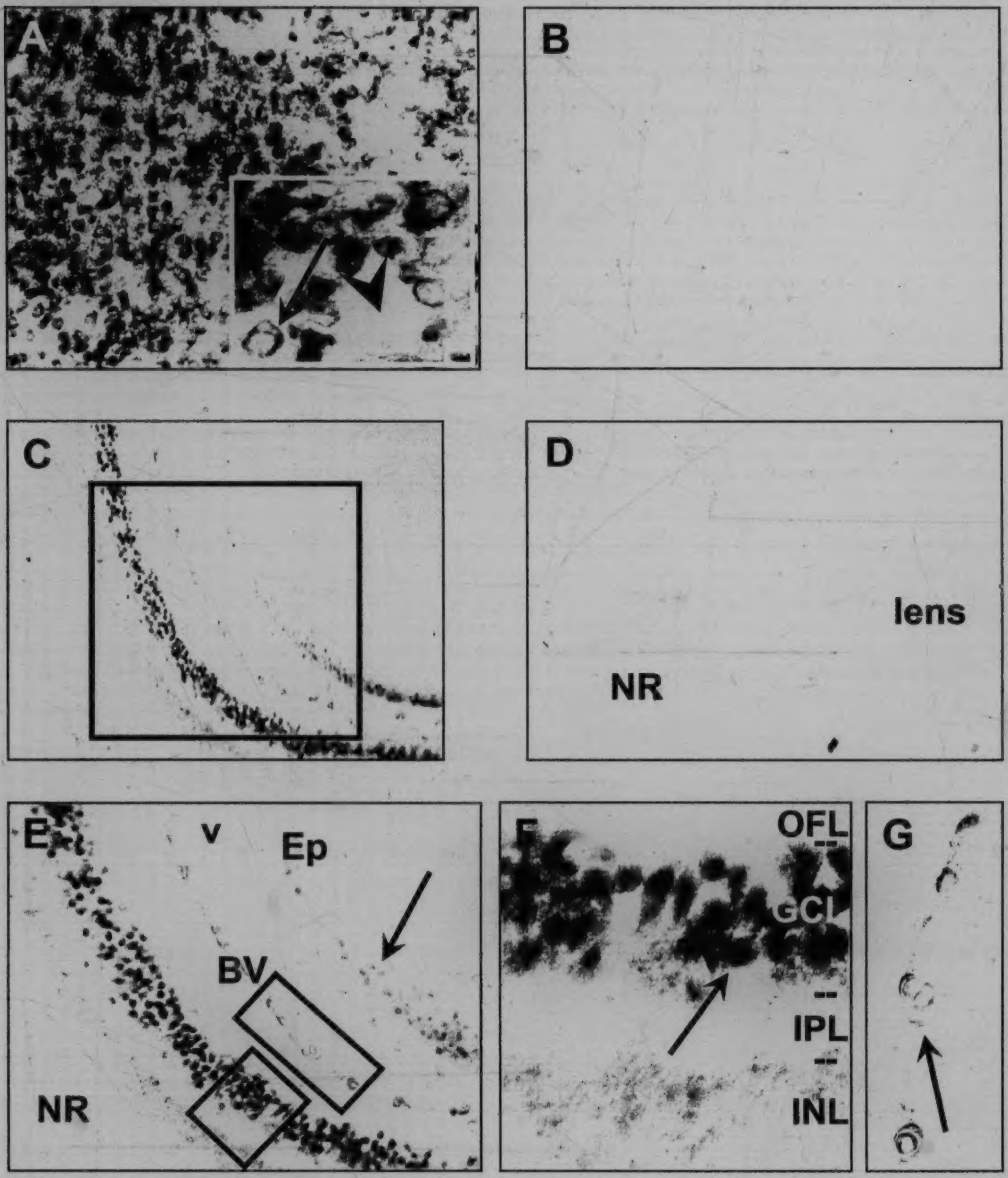
A
B
C
D
lens
NR
E
v
Ep
BV
NR
F
OFL
GCL
IPL
INL
G

Fig. 7.1. *In situ* hybridization using a Dig-labeled riboprobe for growth hormone (GH) mRNA. (**A**) GH mRNA in the cytoplasm of pituitary somatotrophs (arrow, inset). An absence of staining in some pituitary cells was observed (arrowhead, inset). (**B**) Absence of labeling when the antisense probe was replaced by the sense probe in pituitary. (**C**) GH mRNA in a transverse section of a neonatal mouse eye . (**D**) Absence of staining when the antisense riboprobe was replaced by the sense probe in the retina (NR, neural retina). (**E**) Higher magnification of the boxed area in (C) (v, vitreous humor; Ep, lens epithelium; BV, retinal blood vessels). Staining in the epithelial cells of the lens was visible (arrow). (**F**) Higher magnification of the boxed area in (E). Note the strong staining in cells of the retinal ganglion cell layer (arrow, GCL), a faint staining in the innermost cells of the inner nuclear layer (INL) and an absence of signal in the optic fibre layer (OFL) and in the inner plexiform layer (IPL). (**G**) Higher magnification of the boxed area in (E). GH mRNA localized in endothelial cells of retinal blood vessels (arrow). Magnifications: (A), X100; (inset), X1000; (B), X200; (C), X100; (D)-(E), X200; (F)-(G), X1000.

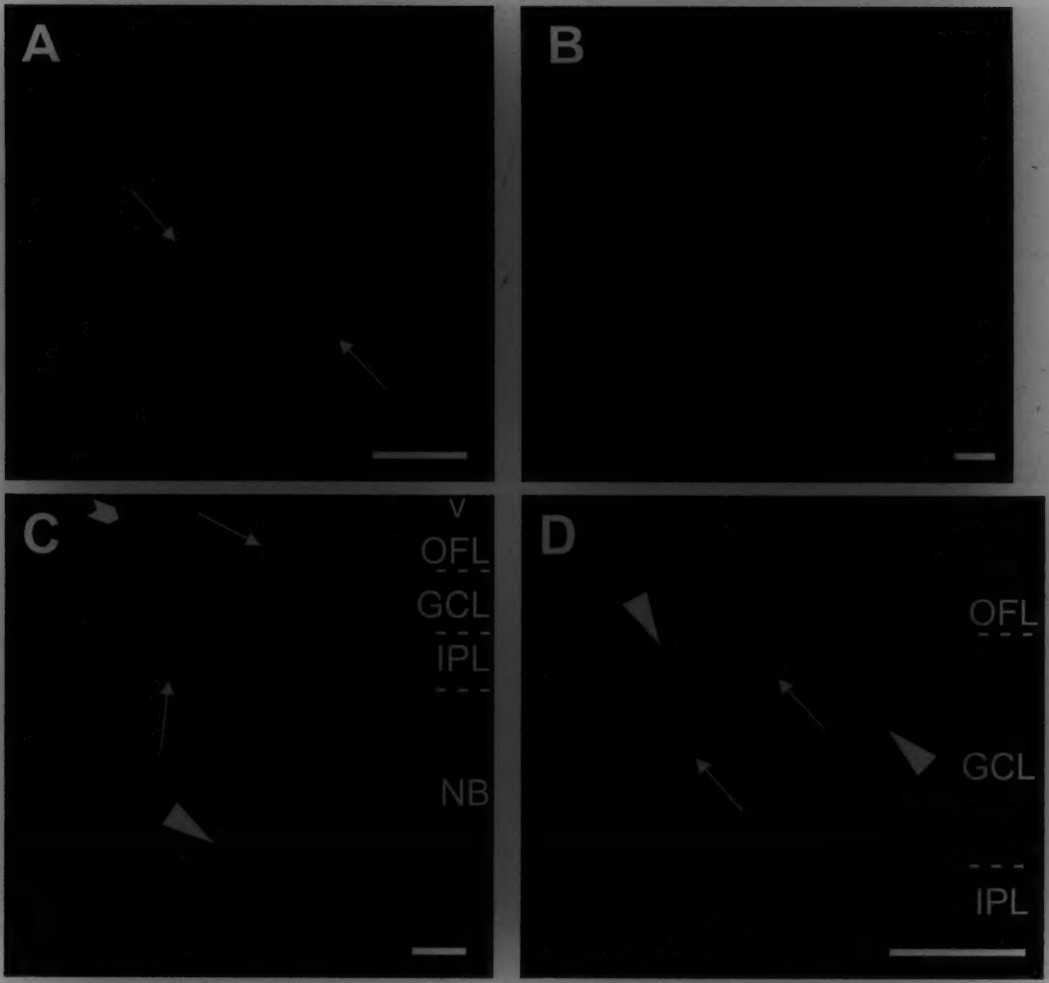

Fig. 7.2. GH immunoreactivity in the adult pituitary and in the neonatal neural retina. (**A**) GH immunoreactivity in the pituitary somatotrophs (arrow) of adult rats. (**B**) Neonatal mouse Negative control (primary antibody replaced by normal rabbit serum). (**C**) GH immunoreactivity in the neonatal mouse neural retina. Note the strong GH immunoreactivity in cells of the ganglion cell layer (GCL), and in the inner plexiform layer (IPL) (arrow). The presence of GH in parts of the optic fibre layer (OFL) (arrow), in blood vessels (chevron) and in cells (arrowhead) within the neuroblastic layer (NB) is also visible (v, vitreous). (**D**) Higher magnification of the inner retina in (C). The presence of GH in the cytoplasm of cells in the GCL (arrows) and in neurites of GCL cells (arrowhead) is shown. Scale bar = 20 μm

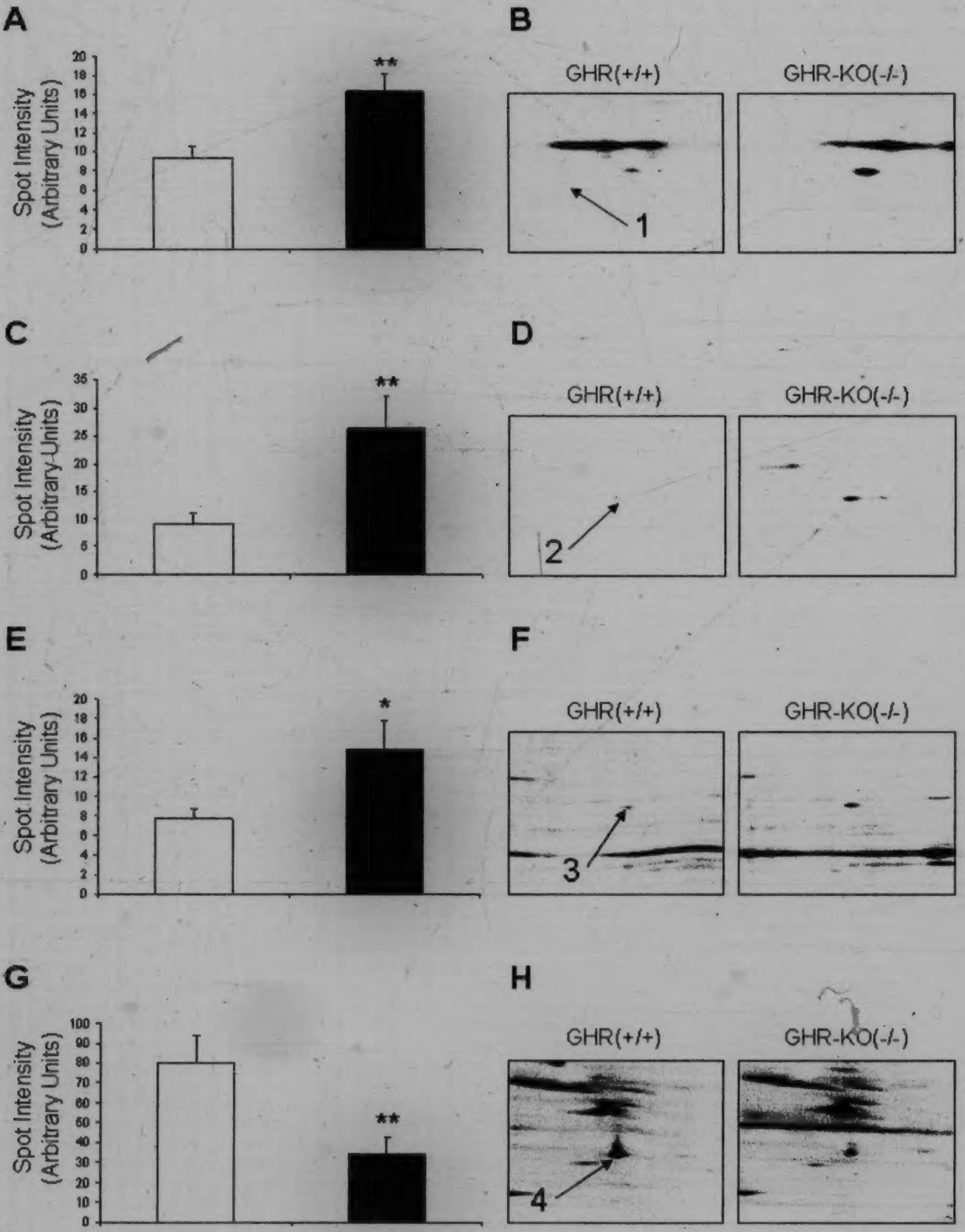
A
B
Spot Intensity (Arbitrary Units)
20
18
16
14
12
10
8
6
4
2
0
**
GHR(+/+)
GHR-KO(-/-)
1
C
D
35
30
25
20
15
10
5
0
**
2
E
F
*
3
G
H
100
90
80
70
60
50
40
30
20
10
0
4

Fig. 7.3. (**A, C, E, G**) Spot intensity (arbitrary units) for identified proteins in the neonatal retina of GHR wild type (+/+) (n=6, open bars) and GHR-KO (-/-) mice (n=6, solid bars) (means + SEM), asterisks indicate significant differences between the groups, * for $p<0.05$ and ** for $p<0.02$ and (**B, D, F, H**) representative 2-DE highlighting individual protein spots (1-4). Protein 1, protein kinase C inhibitor 1 (A, B); protein 2, cyclophilin A (cyclosporine A-binding protein) (C, D); protein 3, signal transduction-associated protein 3 (E, F); protein 4, brain abundant, membrane attached signal protein 1 (G, H).

7.5 References

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Chapter 8

General Discussion

Chapter 8: General Discussion

This thesis addressed the hypothesis that GH is present in tissues of the developing visual system, in which it acts as an autocrine / paracrine growth factor.

8.1 Summary of results

1. GH has been detected in numerous developing nervous tissues of the early chick embryo including the neural retina, using immunohistochemistry (Harvey et al., 2001). The origin of this GH immunoreactivity was however uncertain, since Takeuchi and coworkers (2001) failed to detect the full length GH mRNA while they discovered the presence of a novel GH isohormone, small chicken GH (scGH) transcript, within E17 embryonic chick eyes. Retinal GH immunoreactivity was thus fully characterized in chapter 2. The full length GH mRNA was amplified from the E7 neural retina and was particularly abundant within cells of the ganglion cell layer (GCL). Within the E6-9 retina, GH immunoreactivity was mostly associated with submonomer moieties of 15 and 16 kDa and these moieties were no longer present in the adult neural retina, indicating their involvement in the development of this tissue. Using the same chicken GH antiserum as the one used in our initial study (Harvey et al., 2001), GH distribution was confirmed in E7-8 chick embryo neural retina and found to be mostly abundant in RGCs. Since RGCs project their axons to visual processing centers in the brain, GH immunoreactivity was traced, in chapter 3, within RGC axons and was found to be located throughout the retinofugal tract but also in the tectobulbar tract of the optic tectum, where it colocalized with a neurofilament marker. GH was found in the optic tectum as early as E4, before RGC axon reach this tissue, and GH mRNA was also detected in this tissue, indicating that GH is likely to be locally produced within the optic tectum and not derived from the retina.

2. To demonstrate that GH acts as an autocrine / paracrine growth factor within tissues of the visual system, the distribution of GHR was first examined in chapter 3 then in chapter 4, in sites of GH production at E7, before pituitary differentiation. GHR protein distribution mirrored that of GH within the E7 developing retina, retinofugal tract and optic tectum. GHR within the neural retina and retinofugal tract likely originates

from cells of the GCL where the mRNA was detected. Retinal and tectal GHR were functional, as GHRG-1 cDNA could be amplified in these tissues, and as IGF-1 mRNA levels were upregulated upon addition of GH to retinal explants (chapter 2). In chapter 4, the chronotopic distribution of GH within retinal RGC axons was determined and corresponded to the period of axon elongation, indicating GH might be involved in this process. 10^{-9}M and 10^{-6}M GH treatment was thus shown to promote axon elongation from E7 immunopanned retinal ganglion cells by 135% and 230%, respectively, compared to PBS control. These data, in combination with another study that demonstrates endogenous roles for retinal GH in embryonic chicks (Sanders et al., 2005), indicates that GH is likely to act exclusively as an autocrine / paracrine growth factor within tissues of the visual system in early chick embryo development.

3. The possibility that scGH is also present and translated within the developing retina was next examined. In chapter 5, scGH mRNA was also reverse transcribed and amplified within whole eyes and neural retina of E7 chick embryos. Using an antibody raised against the putative unique N-terminus of scGH, scGH was found to be translated and distributed throughout cells of the neural retina, within a cytoplasmic and subnuclear compartments of RGCs in particular. It was also found in the optic nerve, where it colocalized with a cell marker for glial cell progenitor. scGH and GH distribution appeared to differ within the neural retina, as scGH could not be traced within RGC axons. In E7 ocular tissues, scGH immunoreactivity was mostly associated with a 31 kDa moiety but also with a moiety of approximately 16 kDa, close to its predicted size (16.5 kDa). ScGH was bioactive, as its immunoneutralization promoted cell death of E8 immunopanned RGC culture. The possibility that scGH binds to GHR dimer, despite its severe truncation, was theoretically examined in chapter 6, using a homology modeling approach. scGH appeared to lack the residues essential for binding to the human GHR dimer and might thus act via an alternate receptor (or without a receptor).

4. As the distribution and function of GH within the neural retina of embryonic chicks might be species-specific, the possibility that GH is present and functional within the newborn mouse neural retina was also examined in chapter 7, using the advantage that this species can be genetically manipulated. While GH mRNA was mostly present in cells of the GCL, GH protein was found throughout the neural retina but with a higher

signal in the ganglion cell layer and the inner plexiform layer. In GHR knockout newborn animals, protein kinase C (PKC) inhibitor-1, cyclophilin-A, signal transduction associated protein-3 (SLM-2) were upregulated while brain abundant, membrane attached signal protein-1 (BASP-1) was downregulated. Since these proteins are involved in vascularization, neurite outgrowth, synaptogenesis and cell proliferation, this suggests GH could regulate these processes in the developing rodent neural retina.

8.2 Implications

GH: a multifunctional trophic factor

As mentioned, GH promotes cell survival within the developing neural retina in E6-8 explants, E8 RGC culture and *in ovo* at E6 and E8 (Sanders et al., 2005). In addition, we show here that GH promotes axon growth of immunopanned RGCs at a similar developmental stage (Chapter 4). GH dual action on RGC development suggests that it acts as a neurotrophic factor (i.e. a cell growth and survival molecule affecting neural cells). Ciliary neurotrophic factor (CNTF) and leukemia inhibitor factor (LIF), whose receptors belong to the class I cytokine / hematopoietic superfamily of receptors (like GHR) similarly promote axon growth and prevent cell death in the developing neural retina of rodents (Frade et al., 1999; Jo et al., 1999; Goldberg et al., 2002), suggesting a common roles for cytokines within this neural tissue.

Interestingly, a correlation has been suggested between the ability of a growth factor to promote axon growth and its ability to support RGC survival (Goldberg et al., 2002). Although very little is known about the cause of this correlation, it can be hypothesized that these two processes might require similar upstream but different downstream signal transduction molecules. It is thus of interest that the PI3K / Akt pathway is implicated in both EPO mediated RGC antiapoptotic and regenerating action following optic nerve lesion (Kretz et al., 2005). IGF-1 similarly prevents amacrine cell degeneration and promotes its neurite outgrowth, and these effects are correlated with an increase in Akt phosphorylation (Politi et al., 2001). As GH has been shown to promote cell survival by inducing Akt phosphorylation (Sanders et al., 2006) , it can be hypothesized that axon growth might be similarly mediated by Akt signaling pathway.

In addition, GH might exert its cell survival and axon growth actions on different subcellular locations. GH is predominantly present in the optic fibre layer (OFL) during the period of RGC axon elongation (E4-12) and appears to be subsequently present in inner plexiform layer composed of neurite (E12-18) including RGC dendrites (chapter 4). GH might thus preferentially alter neurite growth by acting subcellularly. Conversely, GH has been detected in the cell soma of RGCs and this presence is detected until at least E18 (chapter 4). GH is thus located in RGC soma throughout the two main waves of apoptosis [E5-E8 and E11-E16 (Mey and Thanos, 1992; Frade et al., 1997)] and might promote its cell survival activity in this subcellular compartment. Although to the best of our knowledge, such compartmentalized function for GH has never been reported, GHR appears to be enriched in subfractions (caveolae and lipid rafts - cholesterol rich plasma membrane microdomains) of the plasma membrane of 3T3 and IM-9 cell lines (Yang et al., 2004). In addition, NGF has been reported to act differentially on the cell soma and on the cell axon in rat sympathetic neurons (Campenot, 1994). Thus, NGF action on neuronal axons promotes cell survival and axon growth, whereas its exclusive action on neuronal cell soma promote cell survival only and axons retract (Goldberg, 2003).

GH: interactions with other retinal growth factors

In addition to GH, many other growth factors are present and act within the neural retina within similar developmental stages. The fact that retinal cells are regulated by so many growth factors is somewhat intriguing. It is possible that some of these molecules act synergistically with GH. Synergistic actions have been previously reported; for instance, BDNF and CNTF induce faster rate of RGC axon elongation together than either separately, suggesting that these growth factors regulate different aspects of axon growth (possibly via different downstream signaling molecules) (Goldberg, 2003). Some of these putative synergistic effects might reflect receptor cross-talk. Interestingly, GH signaling has been shown to stimulate EGF (epidermal growth factor) receptor phosphorylation leading to gene activation in preadipocytes and liver (Yamauchi et al., 1997; Yamauchi et al., 1998; Huang et al., 2003).

The spatio-temporal actions of retinal growth factors may also differ. For instance, GH might preferentially affect the survival of RGCs (Sanders et al., 2005) where its receptor is located (chapter 4), whereas growth factors such as insulin and

BDNF prevents the cell death of proliferating neuroepithelial cells and of young retinal neuroblasts, respectively (Frade et al., 1997; Diaz et al., 1999) during similar developmental periods. In addition, proinsulin, IGF-1, BNDF and NT-3, have been hypothesized to act in a temporal-sequential manner, successively regulating retinal cell numbers (Izumi et al., 1995; de la Rosa and de Pablo, 2000). GH might similarly promote axon growth and survival actions during a restricted window of development (chapter 2, 4).

One can also hypothesize that a regulatory hierarchy exists between these trophic factors; GH actions could thus be mediated by intermediary growth factors. GH has been shown to promote the expression of growth factors that have been involved in the regulation of cell death during the first wave of apoptosis in the developing chick embryo retina and of RGC axon growth in neonatal rodent retina, such as FGF-2 (Izumi et al., 1995; Desire et al., 1998a; Desire et al., 1998b; Goldberg et al., 2002) and BMP-4 (Li et al., 1998; Li et al., 2001). IGF-1 could also promote some of GH actions in the retina, especially as it is traditionally considered to be a mediator of GH action (Le Roith et al., 2001), and as GH has been shown to increase the expression of IGF-1 in E8 retinal explants (chapter 2). IGF-1 has been shown to induce axon outgrowth in combination with 5 μM forskolin in P8 immmunopanned rat RGCs (Goldberg et al., 2002). Its ability to promote axon elongation in chick is however uncertain, since IGF-1 does not induce neurite regrowth from "mature" immunopanned RGCs (Brocco and Panzetta, 2002). Nevertheless, IGF-1 could promote axon growth in other types of RGCs, such as "immature" RGCs. Indeed, IGF-1 induces an increase in G4 levels, a neuroglial cell adhesion molecule (Ng-CAM) found in the OFL (Hernandez-Sanchez et al., 1995) and G4 promotes neurite outgrowth from E6 retinal explant (Morales et al., 1996). In addition, IGF-1 promotes the expression of α6 integrin subunit *in vitro* in dissociated E5 chick embryo retinal cells and *in ovo* in E6 retina (Frade et al., 1996). α6 integrin constitutes, with β1 integrin subunit, a laminin receptor in the retina (de Curtis et al., 1991; De Curtis, 1993) and laminin has been shown to promote axon growth in chick embryo RGCs (Cohen et al., 1986) and in rats, dependently from the α6 subunit (Cohen and Johnson, 1991; Ivins et al., 2004). Taken together, this suggests IGF-1 could mediate GH's action on axon growth.

GH: putative novel roles in visual system development

GH might also be associated with processes of retinal development other than cell survival (Sanders et al., 2005), axon growth (chapter 4) or vascularization (chapter 7) (Hellstrom et al., 2002), including functions that are not classically attributed to neural GH.

GH involvement in cell migration during the development of the nervous system is unknown. However, some evidence suggests GH might be involved in this process within the developing optic tectum, and possibly within the developing retina. GH and GHR have been detected in developing ventricular zone in embryonic chicks and fetal rats, where radial glial cells differentiate (Garcia-Aragon et al., 1992; Harvey et al., 2001; Murphy and Harvey, 2001) and in radial processes of the optic tectum resembling those of tectal radial glia (chapter 3). Glial cells play an important role in cell migration in the tectum, since the processes of radial glia provide a support structure along which neurons migrate (Gray and Sanes, 1991, 1992; Snow and Robson, 1995; Parnavelas and Nadarajah, 2001). In addition, GH and GHR are also detected within the tectobulbar tract, along which tangential migration occurs (Gray and Sanes, 1991; Mey and Thanos, 2000). GH could thus indirectly promote migration by inducing the differentiation of glial cells, as shown in rats (Turnley et al., 2002) and of tectal ganglion cells. GH has also been shown to promote cell adhesion and cell motility in other (non-embryonic) tissues. GH promotes the cell migration of thymocytes and T cells (Taub et al., 1994; Smaniotto et al., 2005). Moreover, autocrine production of hGH induces a plakoglobin-dependant increase in cell motility and invasiveness of mammary carcinoma cell (Mukhina et al., 2004). Taken together, this suggests that GH might directly or indirectly promote cell migration during development.

Nothing is known about the putative roles of GH in axon guidance. During pathfinding, signaling pathways for numerous guidance cues induce actin reorganization within the growth cone, the growing tip of an axon (Dent and Gertler, 2003). GH has been shown to reorganize the actin cytoskeleton in CHO cells stably transfected with GHR (Goh et al., 1997; Zhu and Lobie, 2000) and in 3T3 cells (Herrington et al., 2000). Moreover, GHR mediated signaling promotes the expression of BASP-1 (chapter 7), a molecule that belongs to a family of proteins associated with axon guidance regulation (Mosevitsky, 2005) and that alter the actin cytoskeleton (Frey et al., 2000). In addition axons navigate in a complex environment during their elongation to establish connections with their targets, and molecular cues present in this environment guide growing axons to their targets. Since GH is not located within the path of visual system axons, but within them, GH is unlikely to be a guidance cue *per se*, but might modulate the responsiveness to guidance cues at the growth cone. Another hormone, insulin, has been previously suggested to modulate axon guidance in the drosophila visual system (Song et al., 2003).

Another important mechanism involved in the development of the nervous (visual) system is the establishment of mature cell connections. Although nothing is known about GH involvement in this developmental process, GH might promote synaptogenesis within the retina and possibly within the optic tectum. GH involvement in the establishment of synapses has previously been suggested (Noguchi et al., 1983; Noguchi, 1996), as the levels of thy-1 antigen, distributed primarily in synaptic regions of the rat brain (Watanabe et al., 1981), are decreased in Snell dwarf mice (Noguchi et al., 1983; Noguchi, 1996). In addition, GH regulated BASP-1 has been shown to promote synaptogenesis in mice neuromuscular junction (Caroni et al., 1997). It is also of interest that GH is present within the inner plexiform layer of newborn mice (chapter 7) and of E12-18 chick embryos (chapter 4), at a developmental stage when (or soon before) and where synaptic contacts form (Fisher, 1979; Mey and Thanos, 2000). Moreover, GH might promote intercellular connection via upregulation of connexin-43. Connexin-43 is a molecule forming GAP junctions and is upregulated in the cerebral cortex and hypothalamus upon administration of bGH to adult hypophysectomized rats (Aberg et al., 2000). GH might thus promote synaptogenesis and GAP junction formation in the developing visual system.

GH could also be associated with cell proliferation and cell differentiation as previously shown in other developing nervous tissues (Turnley et al., 2002; Ajo et al., 2003). Overall, this would indicate that GH might be a growth factor with broad range actions leading to a common outcome: retinal growth. Multifunctional retinal growth factors have been previously described. For instance, FGF-2 has been implicated in all aspects of retinal development from the establishment of NR fate within the optic cup

(Pittack et al., 1997) to neurite outgrowth and axon guidance (Holt et al., 2003; Kim et al., 2003). The multiple signalling pathways involved in GH induced signal transduction (Herrington and Carter-Su, 2001) and the different GH variants present in the developing retina (at least in the chick embryo) (chapter 2 and 5) would constitute the molecular basis for this putative multifunctional role.

scGH versus GH

It is of great interest that scGH and GH are both present within the developing neural retina of E7 chick embryos. As hypothesized in chapter 5, the transcription of these two GH isohormones might be driven by different promoters; this indicates that scGH and GH might be differentially regulated. Although nothing is known about the regulation of scGH transcription, GH transcription might be induced by pit-1 or related POU domain transcription factors. Pit-1 induces the transcription of *GH* gene in adult pituitary somatotrophs (Nelson et al., 1988; Fox et al., 1990; Pfaffle et al., 1992) and the possibility that pit-1 regulates the transcription of retinal GH is supported by the presence of pit-1 and pit-1 mRNA within the E7 neural retina of embryonic chicks (Harvey et al., 2003). Interestingly, POU domain transcription factors (POU4f2 and 3) are expressed by RGCs and, like GH [chapter 4 and (Sanders et al., 2005)], contribute to axon growth (Gan et al., 1999; Wang et al., 2000; Wang et al., 2002) and cell survival (Xiang, 1998; Gan et al., 1999) of these cells. It is thus tempting to speculate that POU4f2 and / or POU4f3 might also contribute to the regulation of retinal GH transcription.

Both scGH and GH promote cell death of RGCs upon immunoneutralization of their endogenous native forms (chapter 5, Sanders et al., 2005). It is possible that scGH and GH regulates different aspects of cell death, by altering the levels and / or activity of different pro- and anti-apoptotic molecules. It is thus of interest that both proinsulin and insulin prevent cell death in fetal mice retinal explants via PI3-kinase activation and that insulin but not proinsulin promotes the phosphorylation of Akt (a target of PI3-kinase) (Valenciano et al., 2006).

In addition, it is possible that these two molecules might also have different but complementary actions. The presence of scGH in glial cell progenitors within the optic nerve (chapter 5) and of GH within RGC axons comprising the optic nerve (chapter 3 and 5) suggests that they might both contribute to different facets of optic nerve development [such as myelination – via neuroprotection of oligodendrocyte precursor cells - (scGH) and axon growth (GH)]. It is thus of interest that different alternative splice variants of VEGF, including diffusible and nondiffusible isoforms, regulate retinal vessel enlargement and branching, respectively during the development of the retinal superficial vasculature (Stalmans et al., 2002; Carmeliet and Tessier-Lavigne, 2005).

It is also possible that the respective dominant mode of action of scGH and GH differ, as GH is secreted whereas scGH is not (chapter 2 and 5), indicating different cellular trafficking routes (Fig. 8.1). GH could thus predominantly act as an autocrine, whereas scGH might solely act as an intracrine. GH might also adopt a secondary mode of action as an intracrine, since GH is also found within RGC nucleus, where it colocalizes with GHR (chapter 5). Differential modes of action have similarly been reported for FGF-2 alternative splice variants (Sorensen et al., 2006). High molecular FGF-2 isoforms are not secreted and translocate to the nucleus where they act as intracrines. Conversely, 18K FGF-2 is secreted, binds to a membrane receptor, and induces cytoplasmic signalling and / or is internalized by receptor mediated endocytosis and is subsequently translocated to the nucleus (Sorensen et al., 2006). Interestingly, intracrine FGF-2 acts independently from its receptor by directly binding to nuclear molecules such as the antiapoptotic molecule FIF (FGF-2 interacting factor) (Van den Berghe et al., 2000). As hypothesized, scGH could similarly act in a receptorless manner within the nucleus, especially as its monomer form is unlikely to bind hGHR dimer (chapter 6). The possibility that scGH and GH act together in a bound form is also conceivable due to their common localization within a discrete subnuclear compartment (when assessed in Carnoy's fixed neural retina, chapter 5 and 6) and because both molecules contain motif characteristics of leucine zipper dimers.

Retinal GH: species similarities and differences

The distribution of GH mRNA and protein in the chicken (chapter 2-4) and in rodents (mouse, chapter 7; rats, Harvey et al., 2006) is relatively similar at comparable stages of development (namely E7 / 8 for chicken and P0 for rodents). GH is thus primarily synthesized by cells of the GCL in all species studied, while the protein is predominantly distributed in cells of the GCL and in the OFL. Nevertheless, the retinal GH moieties differs, as submonomer 15 and 16 kDa fragments are detected within embryonic and early posthatched chicks (Chapter 2), while 22 kDa monomer GH is present in the retina of newborn rats (Harvey et al., 2006). A second, truncated GH transcript is also a unique characteristic of embryonic chicks and has yet to be described in rodents.

Interestingly, submonomer GH moieties have been shown to be the predominant GH isoforms in other developing tissues of the chick embryo, such as lungs (Beyea et al., 2005). It might thus be pertinent that these submonomer retinal variants disappear after hatch in chicken but that GH immunoreactivity (associated with monomer GH) is maintained in adult rats, albeit at lower levels (Harvey et al., 2006) and in mice (Harvey et al., 2007), and remains at a constant level in the retina and vitreous throughout weaning (Beharry et al., 2006; Modanlou et al., 2006). This suggests the actions of GH in the retina are strictly associated with retinal development in chicks whereas in rodents, GH might also be associated with the post-developmental maintenance and physiology of this tissue.

Retinal GH is highly likely to exert autocrine / paracrine (and possibly intracrine) actions in embryonic chicks. Although GHR (Garcia-Aragon et al., 1992; Lobie et al., 1993) and GH [Chapter 7 and (Harvey et al., 2006)] are synthesized and present within the developing rodent retina, indicating possible autocrine / paracrine actions for GH, such an exclusive mode of action is, however, doubtful in these mammalian species. Indeed, pituitary GH is present within the rodent systemic circulation from E19 onwards (Strosser and Mialhe, 1975) and the blood ocular barrier is leaky perinatally (Cunha-Vaz, 2004). Exclusive autocrine / paracrine actions of GH, however, cannot be ruled out before the onset of pituitary GH secretion, especially as GH and GHR are present in embryonic rat ocular tissues before E19 (Garcia-Aragon et al., 1992; Harvey et al., 2006).

It is possible that GH exert similar functions in rodents and chicken. Indeed, in chapter 7, GH signaling was shown to involve proteins that have previously been directly and indirectly involved in the regulation of neurite outgrowth such as BASP-1 (Frey et al., 2000; Bomze et al., 2001; Mason et al., 2002), PKC inhibitor-1 (Politi et al., 1998) and SLM-2 (Stoss et al., 2001; Arikan et al., 2002; Li et al., 2003; Andreadis, 2005). In addition, it is possible that GH exerts species specific functions within the developing rodent retina. Contrary to chicken, the retina of rodents is vascularized. The fact that GH regulates molecules involved in vascularization such as PKC inhibitor-1 (Cai et al., 2000), cyclophilin-A (Kim et al., 2004), and SLM-2 (Cohen et al., 2005), strongly supports a role for GH in retinal angiogenesis. Such a role might also affect other mammals with vascularized retina, such as human, since GHR deficient Laron dwarfs and GH deficient children display poorer retinal vascularization (Hellstrom et al., 1999; Hellstrom et al., 2002).

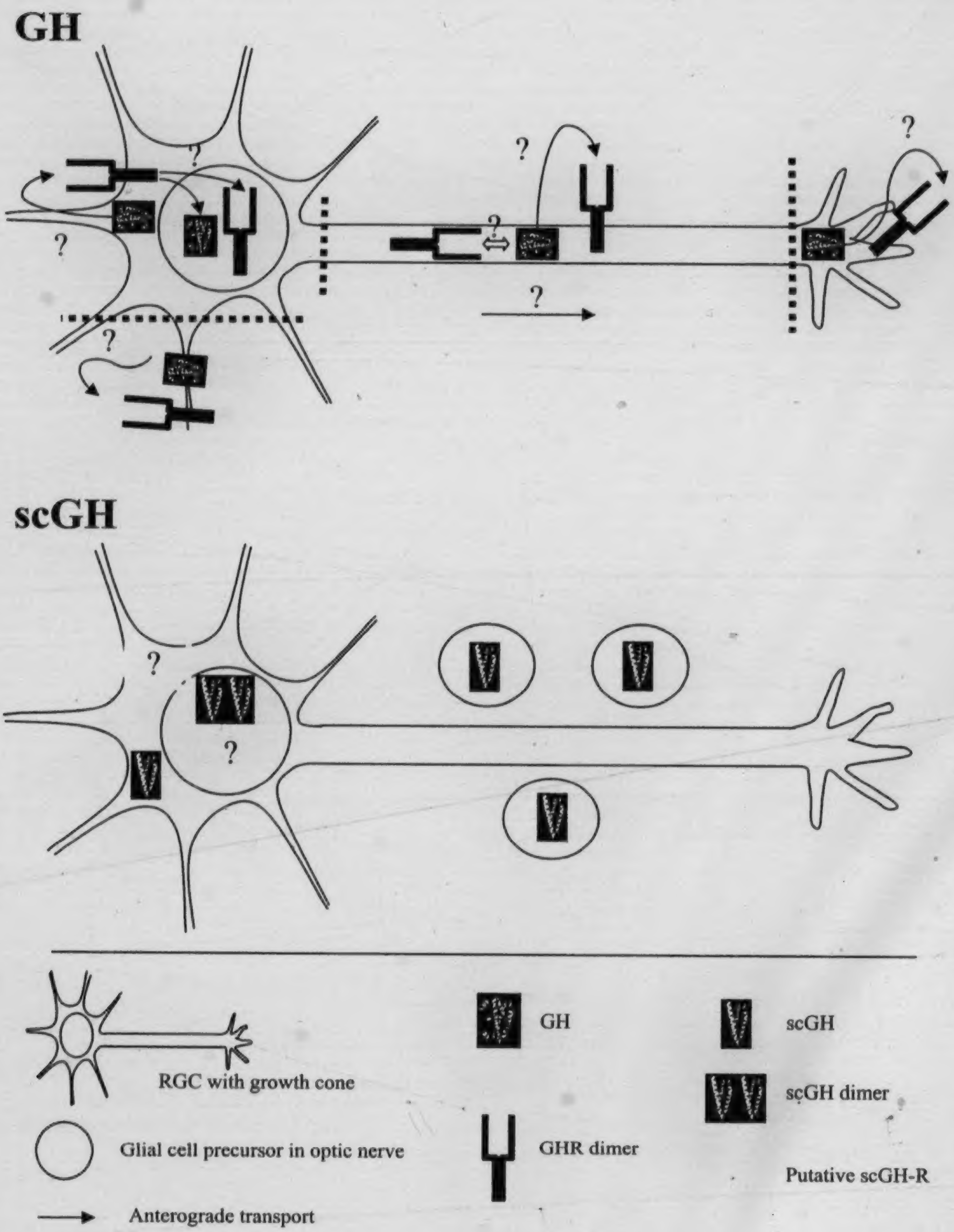
GH in developing rodents: speculative actions

In addition to these functions, GH might exert additional putative functions in rodents. Cyclophilin A is upregulated in GHR knockout animals (chapter 7). Cyclophilin A has been involved in the differential regulation of neuronal versus astroglial cell fate decision of progenitor cells (Urano et al., 2006). This is of great interest, as GH has similarly been shown to promote astroglial over neuronal cell fate in rat neuroepithelial cells (Turnley et al., 2002; Turnley, 2005). It is thus possible that GH might similarly influence the cell fate of retinal progenitor cells within the neuroblastic cell layer of the P0 mouse retina via cyclophilin A downregulation, especially as a single retinal progenitor in P0 rodent retina can produce clones composed of one glial (Müller cells) and one or two neuronal (rod photoreceptors with or without bipolar cells) cell types (Turner and Cepko, 1987).

SLM-2 is also upregulated in GHR knockout mice (chapter 7), suggesting GH signaling promotes the downregulation of this protein. Knockdown of SLM-2 has been shown to influence the splice site selection of VEGF-A in a human kidney cell line by promoting the expression of VEGF isoforms over others (Cohen et al., 2005). In the developing retina, the expression of specific isoforms appear crucial for the ontogeny of the superficial vasculature (Dorrell and Friedlander, 2006). Thus, mice expressing a single isoform and not others display altered development of retinal vascularization (Stalmans et al., 2002; Carmeliet and Tessier-Lavigne, 2005). It is thus possible that GH regulates retinal angiogenesis by coordinating the differential expression of VEGF isoforms via the downregulation of SLM-2. SLM-2 is enriched in brain (Stoss et al., 2001) and it has been shown to be an important regulator of the mRNA processing of tau, a microtubule associated protein, by promoting the inclusion of tau exon 2 and 3 to alternative splice variants (Stoss et al., 2001; Arikan et al., 2002; Li et al., 2003). The tau gene includes 16 exons of which 8 (including exon 2 and 3) are alternatively spliced (Andreadis, 2005). Interestingly, exon 2 and 3 lacking tau isoforms are associated with the nervous system development (Andreadis, 2005) and tau has acknowledged roles in neurite outgrowth and stabilization (Shahani and Brandt, 2002). As tau is present in the retina (Andreadis, 2005), it is possible that GH promotes axon development by inducing the transcription of an exon2/3 lacking tau isoform via the downregulation of SLM-2.

8.3 Conclusion

This work has greatly contributed to the basic understanding of GH in the developing nervous system. It has established, for the first time, that GH is synthesized within neural tissues and that it is abundantly present in projecting neurons of the visual system where it colocalizes with GHR (chapter 2-4). This work has also contributed to the discovery of a novel function for GH: axon growth (Chapter 4) and it has raised the possibility that GH is in fact an important growth factor in nervous system development. It has also confirmed the existence of scGH, a novel GH isohormone and has demonstrated, for the first time, that it is expressed, translated, distributed in ocular tissues of embryonic chicks, and promotes RGC survival (Chapter 5). Theoretical examination of scGH binding to hGHR dimer has raised the possibility that scGH uses an alternate receptor or act without one altogether (chapter 6). Finally, these studies were extended to rodents, where GH was shown to be distributed and functional (Chapter 7), thus laying the foundation for a universal role for GH in retinal development. Roles and mode(s) of action of GH and scGH are, however, still poorly understood (Fig. 8.1) and should be the subject of future investigations.


GH
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scGH
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RGC with growth cone
Glial cell precursor in optic nerve
Anterograde transport
GH
GHR dimer
scGH
scGH dimer
Putative scGH-R

Fig. 8.1. GH and scGH putative modes of actions within RGCs and optic nerve. **(GH)** Although GH trafficking is unknown, GH has been detected within RGC cell soma and nucleus and throughout the length of their axons (chapter 2-3). GHR distribution has been shown to mirror that of GH (chapter 3-4). GH has been suggested to undergo anterograde transport within RGC axons, possibly bound to GHR (chapter 3). Although the subcellular sites for GH action are unknown, it is possible that GH is secreted at the level of the cell soma, along the axon [axon elongation can occur along the axon shaft (Goldberg et al., 2003)], at the growth cone [GH is secreted by the axons tip (synapses) of PC12 cells (Schweitzer and Kelly, 1985)] and on dendrites (later on in development, chapter 4), and might exert its function subcellularly at its sites of secretion and / or alter gene transcription, after binding to GHR. GH would thus act as an autocrine and this differential site of actions might constitute the basis of the various functions reported for GH (chapter 4, Sanders et al., 2005). GH might also undergo receptor mediated endocytosis and translocate to the nucleus where it might act on GHR, as GHR and GH colocalize in RGC nuclei (chapter 4). The possibility that GH acts as an intracrine (without being released) and without receptor (or with an alternate receptor) cannot be ruled out. **(scGH)** scGH has been detected in the cell soma and cytoplasm of RGCs (chapter 5) and possibly exists as a dimer. scGH is unlikely to be release and might thus act as an intracrine. scGH actions might not be mediated by GHR (chapter 6). Rather, scGH might use an alternate receptor and / or act directly by binding to cytoplasmic / nuclear signaling molecules and possibly DNA itself. scGH is present in glial cell progenitor of the optic nerve. scGH could affect the development of these cells (such as promoting their survival) and could thus indirectly induce optic nerve myelination.

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Appendix 1

Knockdown of Chicken Growth Hormone Expression Using RNA Interference Technology

Appendix 1: Knockdown of Chicken Growth Hormone Expression Using RNA Interference Technology

A1.1 Introduction

RNA interference has been emerging as a very powerful research tool to study protein functions (Dykxhoorn and Lieberman, 2005). This approach takes advantage of a naturally occurring cellular pathway that leads to the degradation of mRNA upon the complementary binding of small nucleotides (referred to as siRNA – small-interfering RNA) (Hammond et al., 2001; Hammond, 2005). This relatively new technology appears thus to be an ideal tool to study autocrine / paracrine growth hormone (GH). Indeed, it offers more specificity and flexibility than immunoneutralizing antibodies which are used almost exclusively to investigate local roles for GH (Lattuada et al., 1996; Sabharwal and Varma, 1996; Turnley et al., 2002; Sanders et al., 2005), as siRNAs are directed towards a ~20 bp stretch of nucleotides within a given mRNA target. In addition, it also presents advantages over antisense oligonucleotide technology, since its off-target cytotoxic effects appear less pronounced (Achenbach et al., 2003). An RNA interference approach was thus developed to specifically knockdown chicken GH.

A1.2 Materials and Methods

siRNA-mediated knockdown

Construction of LacZ-cGH expression plasmid. An entry clone was generated by ligating cGH cDNA, obtained from adult chicken pituitaries using RT-PCR and forward (5' GCAACTCTCCCGGCAGGAATG 3') and reverse (5' GCCTCAGATGGTGCAGTTGCTCTCTCCGAA 3') primers, as previously described (Baudet et al., 2003), to pCR8 / GW / TOPO vector (Invitrogen, Burlington, Ontario). The plasmid was then transformed and subcloned, as described above. 100 μg/ml spectinomycin (Sigma, Oakville, Ontario) LB plates and medium were used to grow colonies. The ligation was validated by EcoRI endonuclease digestion. Sequencing was then performed using M13F(-20) (5' GTAAAACGACGGCCAGT 3') and GW2 5′-GTTGCAACAAATTGATGAGCAATT A -3′ primers. 100% homology with the published sequence of cGH was found (Baudet et al., 2003).

A recombination reaction was then performed, according to manufacter's instructions, to obtain pSCREEN-iT / LacZ-DEST / cGH vector (Invitrogen) so that the cGH cDNA would be in frame with the N-terminal *LacZ* ORF (open reading frame). In brief, 123 ng of pCR8 / GW / TOPO / cGH was incubated with 150 ng of pSCREEN-iT / LacZ-DEST vector in TE buffer (pH 8.0) with LR Clonase II enzyme cloning mix (Invitrogen) for 1 hour at 25 °C. 2 μg proteinase were then added to the reaction solution and a 10 min incubation was performed at 37°C. The recombinated pSCREEN-iT / LacZ-DEST / cGH plasmid was transformed into one shot TOP10 chemically competent E.Coli and subcloned using 30 μg/ml chloramphenicol LB plate and medium. The plasmid was subsequently extracted using HiSpeed Plasmid Midikit (Qiagen, Mississauga, Ontario, Canada) and sequenced to confirm that cGH cDNA was in the correct orientation and in frame with the lacZ ORF using LacZ (C-term) forward primer (5'ATT[illegible]GTGGCGACGACTCCTG 3') and TK polyA reverse primer (5' CTTCCGTGTTTCAGTTAGC 3').

Transfection of HEK 293 cells with pSCREEN-iT / LacZ-DEST / cGH and siRNA. HEK 293 cells (kindly donated by Dr. J. Casey, University of Alberta, Canada) were seeded onto 10 μg/ml poly-D-lysine coated 24 well plates and grown for 24 hrs in 500 μl DMEM 5% fetal bovine serum, 5% calf serum, 2 mM L-glutamine (all from Invitrogen) without antibiotics. The cells were then cotransfected at 80%-90% confluence with pSCREEN-iT / LacZ-DEST / cGH and either cGH1-siRNA (R(UUUAGUUUCUCAAACACUC)dTdT)[1] and cGH2-siRNA (r(UUGUUCGUGAACACCUUGC)dTdT)[1] designed by Qiagen. These two siRNA were designed to target cGH exon 4, as this exon is in common with all GH isohormones present in the E7 chick embryo (Takeuchi et al., 2001; Baudet et al., 2003). Stealth siRNA targeting LacZ was used as a positive control and non-silencing siRNA (r(ACGUGACACGUUCGGAGAA)dTdT)[1] (Qiagen) was used as a negative control. 3'-AlexaFluor 488 conjugated cGH1 and non-silencing siRNA were also used to label transfected cells. 150 ng of expression plasmid, 5 pmol of siRNA and 1 μl of Lipofectamine 2000 were mixed with Opti-MEM I, according to manufacturer's instructions (Invitrogen), and the newly formed complex was added to each well with 4 to 6 replicate well per treatment. The cells were lyzed after a 24 hr incubation at 37°C with lysing buffer (25 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0, 10% glycerol and 0.1% Triton-X-100) and placed at -80°C until analysis. The ability to transfect HEK cells were assessed by cotransfecting eGFP expressing vector (kindly donated by Dr. T. Ho, University of Alberta, Canada) and a non-silencing siRNA or pSCREEN-iT / LacZ-GW / CDK2 (Invitrogen) and AlexaFluor 488 non-silencing siRNA.

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[1] Antisense strand is shown

β-galactosidase assay. Each sample was assayed for β-galactosidase activity using the FluoReporter lacZ / Galactosidase quantitation kit, according to manufacturer's instructions (Invitrogen). As the endogenously transcribed LacZ-cGH is translated into a β-galactosidase-cGH chimeric protein, the level of β-galactosidase activity is a measurement of LacZ-cGH mRNA levels. 10 μl of clear cell lysate and 100 μl CUG substrate solution (1.1 mM CUG substrate reagent, 0.1 M sodium phosphate, pH 7.3, 1mM $MgCl_2$, 45 mM β-mercaptoethanol) were added into triplicate wells of a 96-black-walled clear-bottom microtiter plate (Fisher, Edmonton, Alberta) and incubated for 30 min at room temperature in the dark. The reaction was stopped by addition of 50 μl stop buffer (0.2 M Na_2CO_3) to each well.

A β-galactosidase standard curve was generated (Fig. A1.1) to determine the amount of β-galactosidase within the cell lysate. 10 μl of β-galactosidase solution (1 pg to 10 ng β-galactosidase (Sigma,) in 10-fold serial dilutions in 1 mg/ml BSA, 0.1 M sodium phosphate, pH 7.3, 1 mM $MgCl_2$, 45 mM β -mercaptoethanol containing solution) and 100 μl CUG substrate solution were incubated as described above. A linear regression with a coefficient of determination of R^2=0.99 was consistently obtained.

Fluorescence measurement and Statistical Analysis. The fluorescent signal was measured using a fluorescent microplate reader equipped with excitation and emission filters centered at 380 and 460 nm, respectively. The amount of β-galactosidase corresponding to the fluorescent signal was determined and the data obtained were plotted and analyzed using Prism 4.0. Statistical differences were determined by unpaired *t*-test or one-way analysis of variance, where appropriate.

Retinal ganglion cell immunopanning

Retinal ganglion cell (RGC) immunopanning was performed as described in chapter 4 except that the growth medium was used without antibiotics.

Treatment of RGCs. For RNA interference studies, RGCs were transfected with 10 to 100 nM siRNA diluted into 0.5 µl TransIT-TKO reagent two hours post-seeding. In brief, TransIT-TKO was mixed with neurobasal medium and incubated for 10 min at room temperature. 3' AlexaFluor 488 conjugated non-silencing siRNA or cGH1-siRNA were gently mixed to this solution and incubated for an additional 10 min. 25 µl of this complex was then added dropwise to the RGCs and incubated for an additional 24 hrs. 50 µM Q-VD-Oph (R&D Systems Inc., Mineapolis, MN) a broad range caspase inhibitor, was added to RGC culture at the time of seeding to prevent cell death induced by GH withdrawal, as previously used (Sanders et al., 2006). RGC were fixed, submitted to immunocytochemistry as described in chapter 4 and vizualized with a fluorescent microscope (Zeiss) equipped with appropriate lasers.

A1.3 Results

cGH targeting siRNAs induce knockdown in HEK cells overexpressing cGH

Two siRNAs were designed to target cGH exon 4 (Fig. A1.2A). As the siRNA transfection to retinal neurons (RGCs) was relatively low (50-65%), the degree of endogenous GH knockdown was measured using HEK 293 cells transfected with LacZ-cGH reporter gene (Fig. A1.2A). The ability to transfect HEK cells with plasmid and double stranded RNA was first validated using an eGFP expression plasmid or a labelled non-silencing siRNA (Fig. A1.2B). The LacZ-cGH reporter gene encodes for a β-galactosidase-cGH chimeric protein and the level of siRNA mediated knockdown was indirectly measured by the levels of the β-galactosidase activity of this chimeric protein. The LacZ siRNA positive control induced a knockdown of 75% while cGH1- and cGH2-siRNAs induced a knockdown of 90% and 86%, respectively, that were all significantly different ($P<0.001$) from non-silencing transfected siRNA or non-siRNA transfected controls (Fig. A1.2C). Similarly, 488 AlexaFluor labelled cGH1-siRNA induced 93% knockdown in similar culture conditions, compared to labelled non-silencing siRNA controls ($P<0.0001$) (Fig. A1.2C).

cGH targeting siRNA induces endogenous GH knockdown in immunopanned RGCs

The possibility that endogenous GH could alter axon outgrowth was similarly investigated. The ability of labelled non-silencing siRNA to transfect RGCs was then assessed and 55-65% neurite growing RGCs were found to be successfully transfected. GH has been shown to prevent cell death, as withdrawal of endogenous GH induces cell death via caspase-3 activation and PARP-1 cleavage (Sanders et al., 2006). siRNA mediated knockdown of endogenous GH might thus lead to increased cell death as illustrated in Fig. A1.3A, which axiomatically makes any axon length measurements impossible. A broad range caspase inhibitor, Q-VD-OPh, was thus subsequently used, as it has been shown to significantly reduce cell death upon immunoneutralization of endogenous GH in E8 RGC immunopanned cell culture (Sanders et al., 2006), and as the inhibition of the cell death pathway does not alter axon length (Goldberg et al., 2002). As determined qualitatively, cells transfected with non-silencing siRNA (Fig. A1.3B) or non-transfected cells (Fig. A1.3C, chevron) appeared to display high levels of cGH, whereas cells transfected with cGH1 siRNA appeared to display much lower levels of endogenous cGH (Fig. A1.3C, D).

A1.4 Discussion

These data demonstrate that exogenous and possibly endogenous GH were successfully knocked down using siRNA in HEK cells and RGC culture, respectively.

Using two different siRNAs, exogenous GH transfected into HEK cells was knocked down by 90% and 86%, respectively. RNA interference mediated gene silencing of GH has been documented once before in GH3 cells with comparable knockdown efficacy (Ryther et al., 2004). As many as 65% of retinal ganglion cells were transfected with non-silencing labeled siRNA. Although neuronal cells are notoriously hard to transfect, such a high level of siRNA transfection using cationic lipid-based reagent is occasionally obtained *in vitro* (Trulzsch and Wood, 2004; Sosa et al., 2006). Using immunocytochemistry, GH targeting siRNA appeared to knockdown endogenous GH compared to non-transfected cells and cells transfected with non-silencing controls. siRNA mediated knockdown of RGC protein has previously been shown for c-Jun, Bax and Apaf-1 in axotomized cells in rats *in vivo* (Lingor et al., 2005), Opa1 mitochondrial protein in immunopanned RGC extracted from neonate rats (Kamei et al., 2005), amyloid precursor protein (APP) *in vivo* in rats (Herard et al., 2006), p75^{NTR} (p75 neurotrophin receptor) *in vitro* in adult rats (Ahmed et al., 2006). This is thus the first report of the gene silencing of a growth factor in embryonic chick RGCs.

As immunocytochemistry constitutes only a qualitative approach to measure knockdown efficacy in RGC culture, quantitative analysis will be performed using western blotting and / or ELISA. Cell death (phenotype control) and axon length will be measured upon siRNA mediated knockdown of endogenous GH, as compared to non-silencing siRNA controls.

In summary, we describe here GH gene silencing in HEK cells and in RGCs using siRNAs. The significant degree of knockdown achieved in this study suggests it offers technological promise to investigate the roles of extrapituitary GH.

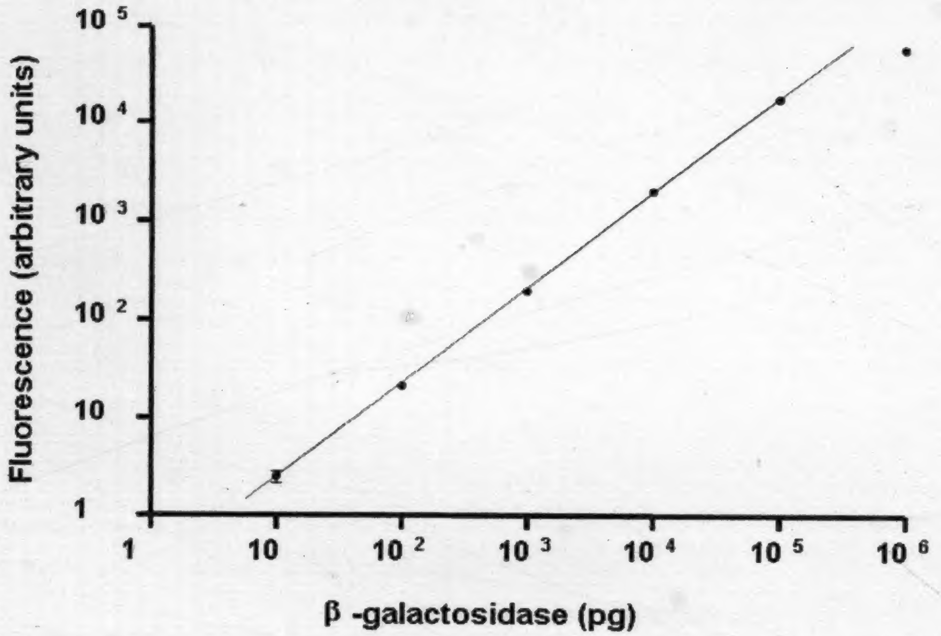

Fig. A1.1. Illustrative standard curve used to determine the amount (in pg) of β-galactosidase in lysed HEK cells. Values are means +/- S.E.M (n=3). The linear regression (with a coefficient of determination of R^2=0.99) is displayed.

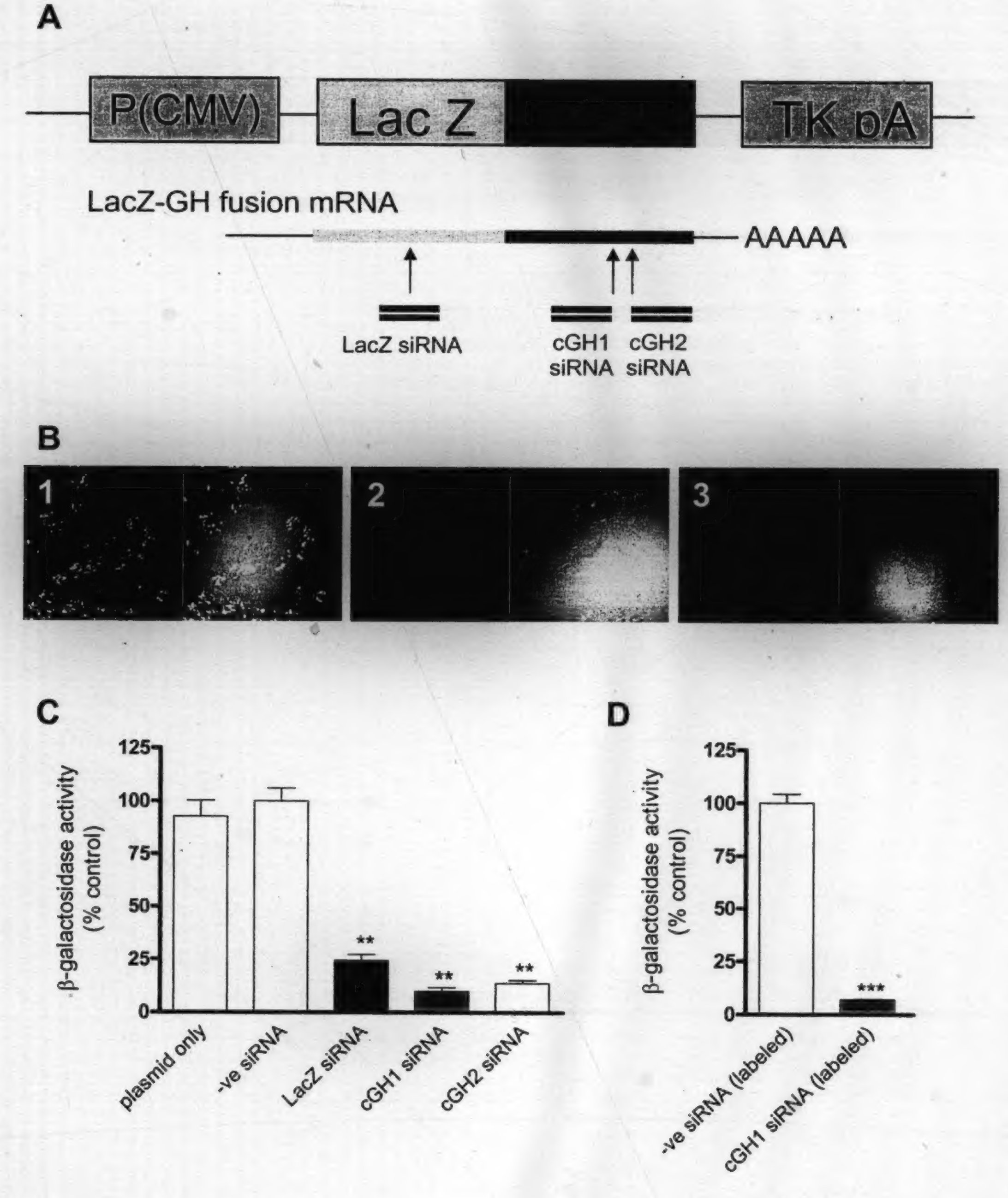
A
P(CMV)
Lac Z
TK pA
LacZ-GH fusion mRNA
AAAAA
LacZ siRNA
cGH1 siRNA
cGH2 siRNA
B
1
2
3
C
β-galactosidase activity (% control)
125
100
75
50
25
0
**
**
**
plasmid only
-ve siRNA
LacZ siRNA
cGH1 siRNA
cGH2 siRNA
D
β-galactosidase activity (% control)
125
100
75
50
25
0
***
-ve siRNA (labeled)
cGH1 siRNA (labeled)

Fig. A1.2. Assessment of cGH siRNA efficacy to knockdown exogenous cGH. (**A**) Schematic representation of lacZ-cGH fusion gene transfected to a human cell line (HEK 293). This gene is driven by the Cytomegalovirus promoter [P(CMV)] and in frame with a polyadenylation site [thymidine kinase poly A (TKpA)]. Upon transcription, a chimeric mRNA is transcribed and targeted by either Lac siRNA or cGH1- and cGH2-siRNAs. (**B**) Assessment of HEK cells cotransfection with (1) eGFP plasmid and non-labeled non-silencing siRNA or (2) pSCREEN-iT / LacZ-DEST / CDK2 and 488 AlexaFluor labeled non-silencing siRNA, (3) as compared to pSCREEN-iT / LacZ-DEST / CDK2 and non-labeled non-silencing siRNA control. (**C**) LacZ, cGH1, or cGH2 siRNA mediated knockdown of β-galactosidase activity of the β-galactosidase-cGH chimeric protein (LacZ encodes for β-galactosidase) compared to non-silencing siRNA (100%) and non-siRNA tranfected controls. Values are means +/- S.E.M. and expressed as a percentage of the non-silencing (-ve) siRNA negative control treated HEK cell, where the control is 100% (*n*=4-6). ** = $P < 0.001$ in comparison with –ve siRNA control (One-way analysis of variance of four experiments followed by Tukey's Multiple Comparison Test). (**D**) Labeled cGH1 mediated knockdown compared to labeled non-silencing siRNA. Values are means +/- S.E.M. and expressed as a percentage of fluorescence obtained in HEK cell transfected with AlexaFluor labelled non-silencing (-ve) siRNA negative control where the control is 100% (*n*=6). *** = $P < 0.0001$ in comparison with –ve siRNA (labeled) control (unpaired *t*-test).

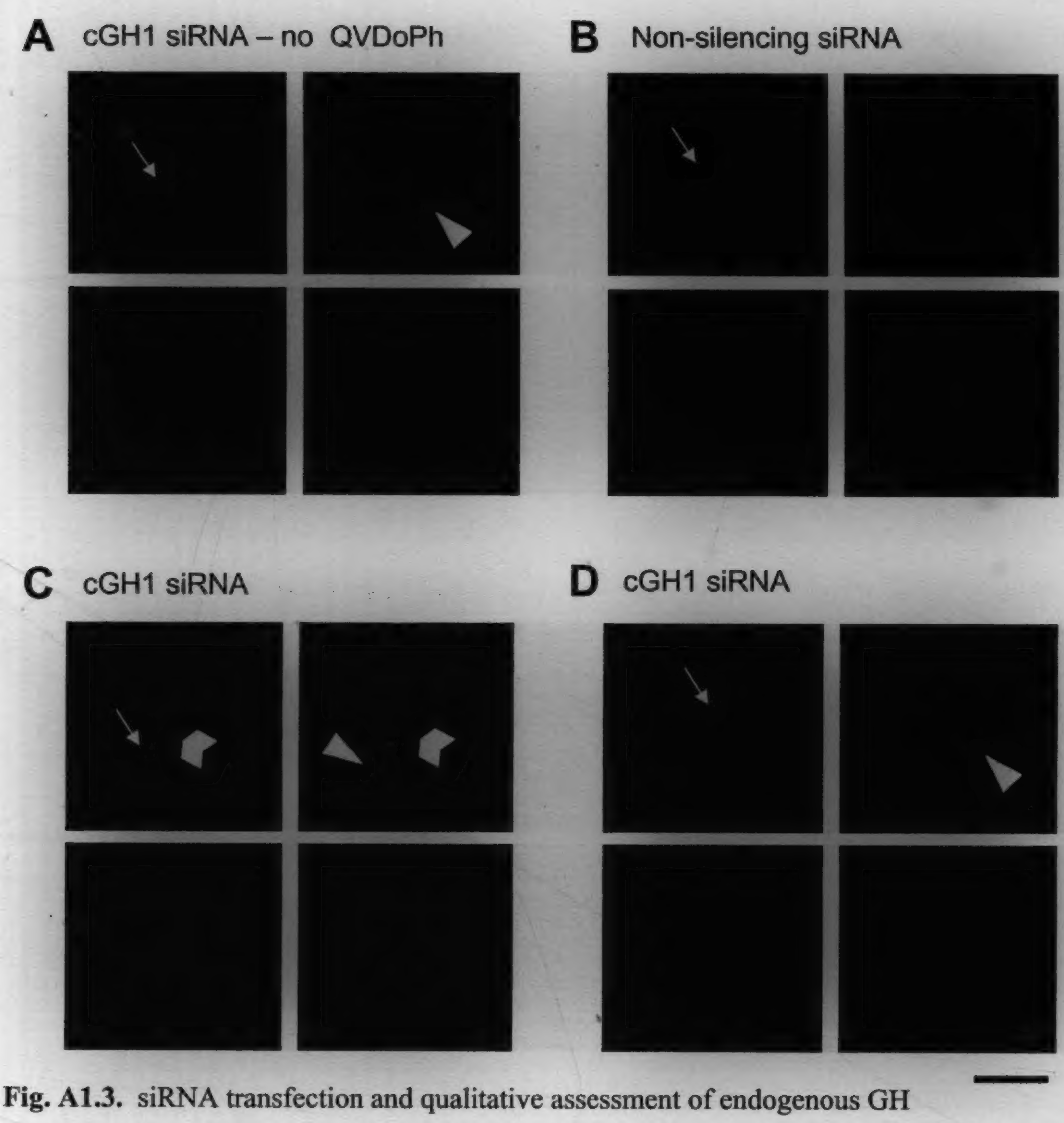

Fig. A1.3. siRNA transfection and qualitative assessment of endogenous GH knockdown. RGCs transfected with 488 AlexaFluor labeled (arrow) siRNA (50 nM) are represented in green, while endogenously expressed cGH is shown in red and RGC nucleus in blue. (**A**) Pyknotic nucleus of RGC transfected with cGH1 siRNA in absence of Q-VD-OPh. Note the faint red cGH signal. (**B**) High expression of GH in non-silencing siRNA transfected cells (arrow). (**C**) Decreased cGH levels (arrowhead) in cell transfected with cGH1 siRNA (arrow). Note the presence of a non-transfected cell with high levels of cGH for comparison (chevron). (**D**) same as (C). Scale bar = 20 μm.

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